VITAMINS AND HORMONES

ADVANCES IN RESEARCH AND APPLICATIONS

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VOLUME I

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Foreword

by Elmer V. McCollum

The invitation of the editors of *Vitamins and Hormones* to write a Foreword to the new publication came as a pleasant surprise, and I am glad to comply. The time is ripe for the founding of such a venture, since it is no longer possible for anyone to read sufficient of the current papers and the library files dealing with these two classes of substances to assimilate all the knowledge which has accumulated. We must increasingly depend upon our colleagues, who maintain mastery of specialized experimentation, to appraise for us the numerous contributions which they alone can interpret, sifting error from truth and assembling scattered data to make a connected account which places a body of related facts in proper perspective. This is the function of the new publication.

It is astonishing that we have progressed so far in so short a time in developing the chemistry and physiology of vitamins and hormones, their sources and the methods for their assay. The vistas of opportunity for further researches in many directions make it clear, that the future is full of promise.

So far as I have been able to discover, the earliest carefully conducted experiments which clearly demonstrated the existence of what are now known as vitamins were those of N. Lunin, a pupil of von Bunge, in Basel, in 1881. He used substances which he believed to be pure, and combined what Prout had, long before, distinguished as the saccharine, the oleagenous, and the albuminous principles, together with certain inorganic salts, to form his experimental diets. He observed that the rapid decline of his experimental animals on these diets could be arrested and a degree of normality re-established by the provision of a small addendum of a natural food, whole milk. He correctly interpreted his observations to mean that there existed essential nutrients which had been hitherto unsuspected. Von Bunge gave wide publicity to his pupil's studies in his text-book of physiological chemistry. For years I sought to learn something of Lunin the man, but without success. It is highly probable that the fertile mind of Professor von Bunge conceived the plan for Lunin's important experiments. Pekelharing, in 1905, repeated in principle the experiments of Lunin and offered a like interpretation of the results. It was not until after the distinguished studies of Hopkins and of Funk were published that vitamin research was launched upon its amazing journey.

In the case of the hormones there has never been any question that the demonstration by Bayliss and Starling, in 1902, of the existence of the chemical regulator, secretin, clearly places them as the fathers of the hormones. The vitamins and the hormones are the most fascinating substances which occupy the attention of physiologists and biochemists of today.

The vitamins participate as components of catalysts which enable the more inert chemical substances, serving as raw materials for the fabrication of living tissues, to undergo the changes which come under the term metabolism. To their peculiar properties are due the fundamental processes of life. The balance and integration among the bewilderingly complex chemical processes of different tissues are in great measure controlled by the hormones. Physically, mentally, sexually and emotionally, we are largely the product of our hormones.

The first volume of Vitamins and Hormones appears at a time when clinicians are cautiously attempting to apply both vitamins and hormones for the benefit of their patients. The future of preventive and of curative medicine is filled with promise in these departments of learning.

The editing of a publication and the preparation of carefully prepared digests of researches by men actively engaged in productive research are labors of love. Workers in many fields of science will be grateful to the editors and to their contributors for furthering the cause of education by giving of their time and labor to the making of the new publication. They will have many appreciative readers and well-wishers.

Editors' Preface

The basic research on vitamins and hormones is conducted by investigators in the fields of organic chemistry, biochemistry, physiology, biophysics and medicine. The results of this research are published in a large number of medical, biological and chemical journals, many of which are not readily available to the scientist or clinician.

To accumulate, correlate and digest the current literature in a field in which research is active and to point out where knowledge is incomplete requires a thorough grasp of the subject. To indicate the directions in which future research would be most fruitful and useful one must have sound imagination. Each chapter in this volume was written by a well-qualified investigator who endeavored to evaluate the present status of his special subject and to indicate what knowledge is lacking.

This volume contains a very complete subject and author index because it is intended primarily as a reference book. With each succeeding volume, "Vitamins and Hormones" will rapidly become a complete reference on all active research in the vitamin and hormone field.

The editors wish to take this opportunity to thank each contributor for the fine manner in which he has accepted and completed his assignment, in spite of the heavy schedules and added responsibilities resulting from the war effort. It is hoped that their efforts will be rewarded by an encouragement of intelligent and useful research in vitamins and hormones,—in the laboratory, and in the clinic.

It is our hope that this is the first of a succession of yearly volumes of Vitamins and Hormones which will chronicle progress and point the way to new achievements.

ROBERT S. HARRIS
KENNETH V. THIMANN

March, 1943
Cambridge, Mass.

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I. INTRODUCTION

The story of choline has many fascinating chapters. The early literature is voluminous because of the relationship of the substance to the ubiquitous phospholipids on the one hand and because of its chemical similarity to neurine, muscarine, and other organic bases of physiological interest on the other. Its nature was a matter of some dispute and its detection and determination have offered many difficulties which still occupy the attention of chemists. The marked physiological activity of its synthetic acetic ester, discovered in 1906 by Hunt and Taveau (1), aroused a new interest in choline which was heightened when Ewins (2) and Dale (3) in 1914 discovered acetylcholine in a natural product, ergot. The brilliant researches of Loewi and of Dale and their colleagues were reviewed by Dale (4) in his Croonian Lectures in which he presented the growing body of evidence that acetylcholine occurs in the animal body and is the chemical agent responsible for humoral transmission of parasympathetic and certain other nerve impulses to effector organs.

It is obviously impossible to discuss the many phases of the choline story adequately in a short review. We propose to limit this presentation to the chemical and nutritional aspects and even then to deal only with the more outstanding contributions.

The older methods of detecting and estimating choline were described by Barger (5) and later reviewed by Zemplén (6) and Sichel (7) in Abderhalden's Handlexikon. An excellent general review of both the chemical and biological aspects is given by Guggenheim (8) in the latest (3rd) edition of "Die biogenen Amine." The physiological significance of choline derivatives has been reviewed by Alles (9) and Gaddum (10).

II. DISCOVERY, NOMENCLATURE, CONSTITUTION AND SYNTHESIS

The name *choline* first appeared in the chemical literature in an article published 80 years ago (11) although previous to that time the compound had been twice isolated. Curiously enough, choline was originally obtained by Strecker (12) from a source in which it is present in very small amount —hog bile (lecithin content from 0.1 to 0.6 per cent, free choline less than 3 mg. per 100 cc., total choline about 0.25 per cent). The first isolation was effected during a study of the mother liquors from the precipitation of the bile acids. Finally a purified and concentrated solution of the base in alcohol was obtained to which chloroplatinic acid was added. The resulting light yellow, flocculent precipitate was found to be easily soluble in hot water, from which solvent the chloroplatinate separated in crystalline form. The free compound was originally described by Strecker as a strong organic base containing sulfur. Thirteen years later, in a further paper on new components of hog bile, he corrected the latter claim, reported an improved technique for isolating the base and named it choline. He gave analyses of the chloroplatinate[1] from which he deduced the empirical formula $C_5H_{13}NO$. The correct formula, $C_5H_{15}NO_2$, differing only by H_2O from that given by Strecker, could not be deduced until the confusing chemistry of ammonia and the substituted ammonias (amines and quaternary bases) and their hydrates had been clarified by Wurtz and Hofmann. It is of historical interest that Strecker suggested "ethylene-oxide trimethylamine" as one possibility for the chemical nature of choline.

The minute amount of choline obtained by Strecker from hog bile and ox

---

	C	H	N	Pt
[1] Found by Strecker	19.4	4.5	4.6	31.6
	19.6	4.5		31.9, 31.9
Calculated for chloroplatinate of $C_5H_{15}NO_2$	19.5	4.5	4.5	31.6

bile undoubtedly originated in lecithin which was hydrolyzed by the baryta treatment involved in the isolation. This was demonstrated clearly by Dybkowsky (13). Strecker does not seem to have realized this although he made the pertinent observations in his second paper that the glycerophosphoric acid which he also found did not occur in the original extract but appeared only after boiling with baryta, and that barium salts of the fatty acids were formed simultaneously.

In 1852, Babo and Hirschbrunn (14) isolated a new organic base by boiling the alkaloid derived from white mustard (*Sinapis alba*) seed with alkali. They called the base sinkalin to indicate its origin from the alkaloid sinapin upon treatment with alkali. Fifteen years later it was shown by Claus and Keesé (15) to be identical with the base isolated by Strecker from bile. Thus Babo and Hirschbrunn were really the first to isolate pure choline although they did not so denote it. Strecker's designation, *choline*, although not assigned until ten years later, came to be generally accepted.

The nomenclature and even the very nature of these strongly basic organic compounds was temporarily confused by Liebreich (16) who isolated an apparently new base from hydrolyzed brain "protagon" (lecithin). Liebreich called the base "neurine." His analytical data (due to impurity of the chloroplatinate) led to the formula $C_5H_{13}NO$, corresponding to a vinyl-trimethyl-ammonium hydroxide. Acceptance of Liebreich's analytical data led to association of the name *neurine* with the vinyl compound. The identity of Liebreich's base from "protagon" with choline from bile was almost immediately suggested by Baeyer (17) and established by Dybkowsky (13). The name "neurine" for this base was eventually dropped although a relic persisted for some time in the modified form of "bilineurine", proposed by Liebreich (18) to distinguish it from the corresponding vinyl base with which it was at first confused. Subsequently the term *neurine* was reserved for vinyl-trimethylammonium hydroxide, a very poisonous base resembling choline and derived from it by spontaneous decomposition (19) as well as by chemical treatment. The vinyl compound was actually prepared synthetically by both Hofmann (20) and Baeyer (17) before it was recognized as a decomposition product of a natural substance.

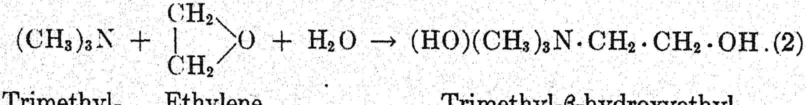
Clarification of the situation began in 1866 when Baeyer (17) isolated a more highly purified product from brain "protagon" and studied its chemical behavior. Treatment with excess concentrated hydriodic acid (Equation 4) converted the base to a substance containing two atoms of iodine, one of which was easily replaced (behaving like that in potassium iodide), the other was removed by digestion with moist silver oxide, *apparently* giving back the original "neurine" (Equation 5). The behavior was similar to that of a dibromo compound prepared 8 years previously by Hofmann (20) from trimethylamine and ethylene bromide (Equation 3).

Baeyer repeated Hofmann's preparation, obtaining a substance which, like the product from "neurine," was convertible by moist silver oxide to a compound apparently identical (analyses of chloroplatinate) with "neurine." This behavior seemed to establish the base from "protagon" as hydroxyethyl-trimethyl ammonium hydroxide, and Baeyer suggested that the choline from bile and sinkalin from white mustard seed are identical with it. However, the somewhat variable analytical data on several different chloroplatinate preparations made it appear possible that "neurine" was a mixture and that both hydroxyethyl and vinyl bases were present. Baeyer observed that the finding of this compound in products as diverse as bile, white mustard seed, and brain indicates a wide distribution in nature and that the compound would therefore probably prove to be of considerable metabolic importance.

The elegant yet simple synthesis of "*névrine*" (*i.e.* Liebreich's "neurine" = choline) by Adolf Wurtz (21) in 1867 was a classical preparation. Acting upon the suggestion of Baeyer that "neurine" was the hydroxy-ethyl base, Wurtz effected the first direct unequivocal synthesis of choline (as chloride) by warming trimethylamine with ethylene chlorhydrin in a sealed tube on a water bath for twenty-four hours (Equation 1). On cooling, a mass of beautiful, prismatic, colorless crystals formed. This chloride was converted to both the gold and platinum double salts and into the free base. The properties of the chloride agreed with those of a sample of a natural material obtained from Liebreich, as did those of the aurichloride, platinichloride and free base made from the synthetic product. Shortly afterwards, Wurtz (22) obtained free choline directly by treating a concentrated aqueous solution of trimethylamine with ethylene oxide at ordinary temperature (Equation 2).

$$(CH_3)_3N + ClCH_2 \cdot CH_2 \cdot OH \rightarrow Cl(CH_3)_3N \cdot CH_2 \cdot CH_2 \cdot OH \quad (1)$$

Trimethyl-amine — Ethylene chlorhydrin — Trimethyl-β-hydroxyethyl ammonium chloride
CHOLINE CHLORIDE



$$(CH_3)_3N + \begin{matrix} CH_2 \\ | \\ CH_2 \end{matrix}\!\!>O + H_2O \rightarrow (HO)(CH_3)_3N \cdot CH_2 \cdot CH_2 \cdot OH \quad (2)$$

Trimethyl-amine — Ethylene oxide — Trimethyl-β-hydroxyethyl ammonium hydroxide
CHOLINE

Almost simultaneously, Baeyer (23) reported further work on Liebreich's "neurine" which finally cleared up the earlier chemical difficulties. Although his synthetic efforts in 1866 seemed adequate proof of the structure, a possibility remained that the treatment with moist silver oxide might have converted the halogen compounds to a product with a vinyl rather than a hydroxyethyl group. Baeyer discovered that the variable analytical data obtained in his studies of the chloroplatinates of the synthetic base were due to the unsuitability of these very soluble and hygroscopic crystals. Utilizing the less soluble aurichloride, which was obtained as a beautifully microcrystalline yellow salt, he found that the one made from the natural material possessed the correct composition for one derived from trimethylhydroxyethyl ammonium hydroxide. However, a careful reinvestigation revealed that heating the di-iodo or di-bromo compounds with an excess of moist silver oxide did indeed convert them to the vinyl compounds (Equation 5).

$$(CH_3)_3N + BrCH_2\cdot CH_2Br \rightarrow Br(CH_3)_3N\cdot CH_2\cdot CH_2Br \quad \ldots(3)$$

Trimethyl-amine — Ethylene bromide — Trimethyl-β-bromethyl ammonium bromide

$$(HO)(CH_3)_3N\cdot CH_2\cdot CH_2\cdot OH + 2HI \rightarrow I(CH_3)_3N\cdot CH_2\cdot CH_2I + 2H_2O \quad ..(4)$$

Liebreich's "Neurine" (*i.e.* Choline) — Trimethyl-β-iodo-ethyl ammonium iodide

$$I(CH_3)_3N\cdot CH_2\cdot CH_2I + Ag_2O \rightarrow (HO)(CH_3)_3N\cdot CH{:}CH_2 + 2AgI \quad ..(5)$$

Trimethyl-vinyl ammonium hydroxide
NEURINE

Baeyer found vinyl-trimethyl ammonium hydroxide and its derivatives to differ from Liebreich's "neurine" and explained the previous confusion by finding that the chloroplatinate of the unsaturated base crystallized with one molecule of water which was not completely removed at the drying temperatures ordinarily used. Proof that "water of constitution" (and not of crystallization) occurred in natural "neurine" was ingeniously established when Baeyer showed that treatment of "neurine" chloride with acetyl chloride gave a new basic substance forming a gold salt less soluble than that of the parent substance and whose composition agreed with that of a compound derived from "neurine" by replacing one hydrogen atom with one acetyl group (Equation 6).

$$Cl(CH_3)_3N\cdot CH_2\cdot CH_2\cdot OH + CH_3\cdot CO\cdot Cl \rightarrow Cl(CH_3)_3N\cdot CH_2\cdot CH_2\cdot O\cdot CO\cdot CH_3 + HCl \quad ..(6)$$

ACETYLCHOLINE CHLORIDE

Baeyer also found that the chloroplatinate of this acetylated base is much less soluble in water than is that of "neurine." Thus Baeyer confirmed what Wurtz had already established—the presence of an alcoholic hydroxyl group in natural "neurine" (choline).

Choline has since been prepared synthetically in several ways, starting from the easily made trimethyl-(β-bromethyl)-ammonium bromide. The conventional treatment with silver oxide and water leads to loss of hydrogen bromide and formation of the vinyl base, but Bode (24) effected the conversion by boiling the bromide for 8 days with a solution of silver nitrate. Krüger and Bergell (25) simplified and shortened the preparation by heating trimethyl-bromethyl-ammonium bromide with an excess of water in a sealed tube at 160° for four hours. The resulting choline bromide was converted to chloride in the usual way by digesting with silver chloride. Lucius and Thoms (26) treated the bromo compound with alcoholic potash at 120° for one hour. A mixture of choline and neurine resulted. Upon evaporation, to remove alcohol, and treatment with dilute hydrochloric acid, neurine chloroplatinate was precipitated by adding platinum chloride and the choline double salt was obtained from the mother liquor.

Synonyms

In the older literature choline is referred to by various names which have since been dropped: the origins of the terms sinkalin, neurine, and bilineurine have already been described. Impure samples of choline have been mistaken for new bases and names, usually derived from the biological source of the material, have been assigned which were later dropped. During a study of the seeds of the beechnut tree (*Fagus sylvatica*) Habermann (27) isolated a base which he believed to be identical with a similar material previously isolated and called fagin by Buchner. The base of Buchner and Habermann was apparently choline. Harnack (28), in the course of a study of the poisonous mushroom fly agaric (*Amanita muscaria*), isolated muscarine and also found an apparently new base called "amanitin." Several years later its nature was established when Schmiedeberg and Harnack (29) prepared some synthetic choline and found amanitin to be identical with it. Gossypin, luridin, and other imperfectly characterized basic materials from plant and animal sources are probably impure choline.

III. PROPERTIES OF CHOLINE AND ITS MORE IMPORTANT SALTS

Choline ($C_5H_{15}NO_2$; mol. wt. = 121.13) can be obtained with difficulty as a colorless crystalline mass although it is usually found in a syrupy state owing to the extraordinary hygroscopicity of the compound. Griess and Harrow (30) first prepared the crystalline form, describing it as a colorless, odorless compound with a caustic bitter taste, readily decomposing at elevated temperatures to give off trimethylamine. Because of its marked tendency to absorb water and carbon dioxide, Guggenheim (31) suggested that probably the crystalline material of Griess and Harrow was the carbonate, but Meyer and Hopff (32) found the crystals easy to obtain if a concentrated aqueous solution be left in high vacuum over phosphorus pentoxide.

When pure choline is heated it neither melts nor distills but breaks down into trimethylamine and glycol. Small amounts of β-dimethylaminoethanol and dimethyl-vinylamine are also formed (32).

Choline is extremely soluble in methyl and ethyl alcohol and in formaldehyde as well as in water. It is only very slightly soluble in dry amyl alcohol, dry acetone and chloroform. Choline is insoluble in dry ether, petroleum ether, benzene, toluene, carbon bisulfide and carbon tetrachloride (33) (34). It is therefore not extracted from alkaline aqueous solutions by organic solvents in more than traces, except by amyl alcohol. Thus it does not go over into the alkaloid fraction in the Stas-Otto or Dragendorff procedures for isolation of these compounds. Moist ether dissolves traces of choline and ether containing phospholipids will dissolve significant amounts of choline (35).

Choline is a strong base, liberating ammonia from its salts and precipitating, as hydroxide, (from aqueous solutions) the salts of the heavy metals. Its solutions dissolve fibrin and prevent the coagulation of proteins.

Dilute aqueous solutions on long standing have often been observed to give rise to neurine although this does not occur in concentrated solutions. The change is due to micro-organisms (literature given by Gulewitsch in 33).

The behavior of choline solutions on warming has been the subject of several studies. Wurtz (36) found dilute solutions to be stable to heat but that concentrated solutions gave off trimethylamine when boiled, leaving an oily liquid, boiling above 190°, which he believed to be glycol. Nothnagel (37) found that up to 4 per cent concentration there was little detectable break-down but in stronger solutions decomposition did occur with formation of trimethylamine, a base (perhaps neurine), an aldehyde-like substance and a non-volatile, water-soluble material. Solutions acidified with hydrochloric acid are more stable than the free base when heated. Roman (34) has reported that during evaporation of very dilute solutions on the water-bath losses occur; if at least 0.3 mg. are present in the final cc. the loss on taking to dryness is usually less than 10 per cent; with more dilute solutions it increases and with less than 10γ may reach 100 per cent. Evaporation in vacuo (300 mm. Hg) leads to smaller losses but extremely dilute solutions cannot be so treated and still give quantitative results. He found pure, dry choline to decompose even at 40° under reduced pressure (300 mm. Hg). Choline chloride does not decompose appreciably even at 180° (36). Gulewitsch (33) studied the effect of heating choline in alkaline solutions. Aqueous baryta and alcoholic solutions of sodium ethylate have been used in the isolation of choline and the effects of both were therefore investigated. He found only a negligible breakdown after boiling choline either in baryta solution for six hours or in 5 per cent sodium alcoholate for twenty-four hours.

All choline salts prepared to date are very soluble in water except the periodide, phosphotungstate, phosphomolybdate, reineckate, and the double salts with gold chloride, with mercuric chloride and with Mayer's reagent (potassium mercuric iodide) and Dragendorff's or Kraut's reagent (potassium bismuth iodide).

The chloride, nitrate, sulfate, carbonate, acetate, oxalate, picrate, and picrolonate are readily soluble in both water and alcohol. The monophosphate, chloroplatinate, acid tartrate, and rufianate are soluble in water but insoluble in alcohol. The double salts with cadmium chloride and with zinc chloride are soluble in water but insoluble in alcohol. The useful properties of several of the more important salts are briefly given in the following paragraphs.

Chloride: ($C_5H_{14}ON \cdot Cl$) extremely soluble in water, deliquescent; very soluble in absolute ethyl alcohol (distinction from betaine), in methyl alcohol and formaldehyde. Less soluble (but significantly more so than free choline) in acetone, chloroform, and carbon tetrachloride. Insoluble in ether, petroleum ether, benzene, toluene, carbon bisulfide.

Chloroaurate: ($C_5H_{14}ON \cdot Cl \cdot AuCl_3$) sparingly soluble in water; very insoluble in alcohol. Deep yellow needles (from hot alcohol) or octahedra and cubes (from very dilute alcohol). M.p. 243–244° (slow heating); 249° (rapid heating) (38), 257° (39), 267–270° (40).

Chloroplatinate: ($(C_5H_{14}ON \cdot Cl)_2PtCl_4$) moderately soluble in water; very insoluble in alcohol (difference from ethanolamine). Dimorphous: crystallizes from hot (1:1) alcohol-water in cubes and octahedra, but from water in monoclinic rhomb-shaped crystals or six-sided pyramids. Both forms of crystal are anhydrous, orange-red in color, stable in the dry state but interconvertible by recrystallization from suitable solvents. The conversion of the isotropic crystals (from alcohol) to the anisotropic forms (by dilution with water and evaporation) may be readily followed under a polarizing microscope. This behaviour distinguishes the chloroplatinate of choline from those of potassium, ammonium, trimethylamine, and neurine, and is of great value for identification purposes (41). The melting point (decomposition) is not characteristic; by quickly heating 241–242° (32); by slower heating, temperatures from 209° to 235° have been noted.

Mercuric Chloride Double Salt: ($C_5H_{14}ON \cdot Cl \cdot 6HgCl_2$) insoluble in cold water; sparingly soluble in hot water; very insoluble in alcohol (difference from ethanolamine). M.p. 249–251° (33), 242–243° (42).

Periodides: Iodine in potassium iodide solution precipitates choline (30) as a very insoluble oil or crystalline material depending upon the conditions (43).

Hexaiodide: ($C_5H_{14}ON \cdot I \cdot I_5$)—black greenish-iridescent oil with strong metallic lustre obtained when potassium tri-iodide solution is dropped into an excess of choline chloride. Very insoluble in water; soluble in alcohol and potassium iodide solution. On contact with powdered iodine, or treatment with KI_3 solution, is converted to the crystalline ennea-iodide.

Ennea-iodide: ($C_5H_{14}ON \cdot I \cdot I_8$)—glistening green needles, very insoluble in water; very soluble in alcohol and concentrated potassium iodide solution. Obtained by treating choline chloride with an excess of potassium tri-iodide; the cloudy precipitate which forms immediately, changes, within a few minutes, into green crystals. In the air they lose iodine rapidly and go over to the oily hexaiodide. On boiling in water iodine is lost and choline iodide is formed (43).

Reineckate: ($C_5H_{14}ON \cdot C_4H_7N_6S_4Cr$) very insoluble in water, dilute hydrochloric acid, dilute ammonia, 0.1 *N* sodium hydroxide; insoluble in absolute alcohol, ether and benzene; soluble in acetone (44) (45) (46) (47). In the presence of excess ammonium reineckate the slight solubility in water is greatly depressed (46).

IV. Detection and Estimation of Choline

No highly sensitive color test for choline is known. Rosenheim (48) noted the development of a reddish-violet coloration upon evaporating a saturated aqueous solution of alloxan with a dilute aqueous solution of choline or its salts; upon adding alkali the color changes to blue-violet. Since the reaction is given also by ammonia, amino acids and proteins (49) and has a limit of sensitivity of about 0.04 per cent it is unsuitable for the estimation of choline in tissue extracts.

As early as 1910, Kauffmann (50) noted that choline could be detected by the characteristic odor of trimethylamine which was given off upon heating with concentrated solutions of sodium or potassium hydroxide. The test is a sensitive one, the odor of trimethylamine being recognizable in dilutions as great as 1:2,000,000, but the reaction lacks specificity since many betaines behave similarly.

Sanchez (51) has described the formation of iodoform (recognized by its odor and reactions with aniline and resorcinol) when choline is heated with iodine and strong sodium hydroxide. The reaction is, of course, quite lacking in specificity.

There are several very sensitive although non-specific precipitants for choline. At a dilution of 1:2,000,000 choline in alcoholic solution may be detected by the precipitation (on standing) of double salts with platinum, gold, and mercuric chlorides (52). In water the reactions are much less sensitive, that with platinum chloride failing completely. In aqueous solution potassium tri-iodide gives a precipitate at dilutions from 1:20,000 (53) to 1:2,000,000 (54) depending upon the conditions (55) (56); reinecke salt gives a precipitate at 1:50,000 (57), phosphotungstic acid at 1:20,000 (53), and phosphomolybdic acid at 1:10,000 (53).

Choline gives precipitates with potassium mercuric iodide (Mayer's reagent) and with potassium bismuth iodide (Dragendorff's or Kraut's reagent). Schoorl (58) discusses the use of these precipitates, of the salts with picric and picrolonic acids, of the double salts with platinum chloride and gold chloride, and of the periodide for the detection of choline under the microscope.

Quantitative micro-chemical procedures for determining choline in biological materials depend upon either the insolubility of choline periodide and reineckate or the breakdown of choline to trimethylamine under the influence of strongly alkaline solutions.

The periodide separation is generally considered to be the most sensitive of the various procedures for precipitating choline. Although Griess and Harrow (59) had utilized the insolubility of the periodide to isolate choline as early as 1885, the procedure as now used apparently had its origin in 1896 when Dr. Florence of Lyon first described a medico-legal test for semen stains (60). This test, based upon the formation of typical crystals when the suspected material was treated with iodine dissolved in potassium iodide solution, was subsequently shown to depend upon the insolubility of choline periodide.

Within a year, Richter (61) noted that other substances than semen gave the Florence reaction and Struve (62) also observed the reaction in many human secretions where semen could not be present as a contaminant; moreover, Struve got a positive reaction from egg yolk (hydrolyzed) but not from egg white, and found it in the juice expressed from various parts of many plants (hyacinth flowers, flowers and leaves of the rose and linden, etc.), and in all the wines which he examined. When Struve found that the substance responsible for the test occurs both free and combined in almost all plant and animal tissues, the free form being water-soluble, the combined forms being ether- and alcohol-soluble, he suggested that choline was the causative agent, but he considered the crystals obtained in the test too unstable for isolation and investigation.

Bocarius (63), however, devised a procedure for obtaining a sufficient quantity of the crystals to prove by chemical identification that choline was indeed the agent responsible for the test. Human semen, preserved with 2 per cent formalin, was cleared with lead acetate, filtered, freed of lead and concentrated to one-third its volume. The solution was slightly acidified with hydrochloric acid and treated with sodium tri-iodide. After several hours the large crop of typical Florence crystals was centrifuged down, washed with ice-cold water, treated with silver oxide to free the base and finally converted to the chloroplatinate. Suitably purified, this was found to be identical with the well-known choline chloroplatinate (Pt found 31.65 per cent; theory 31.64 per cent).

Rosenheim (48) combined the periodide reaction on a microscope slide with the chloroplatinate precipitation in alcohol (cf. Donath, (64)) as a characteristic test for choline in biological materials.

Booth (55), in a re-investigation of the periodide test for choline, gives the limiting dilution for the test on a microscope slide as 1:50,000 and says that while acetylcholine never gives a crystalline precipitate, tiny black granules can be detected to a dilution of about 1:10,000. The water-soluble choline precursors found in brain, kidney, and liver do not give a precipitate with potassium tri-iodide.

Kahane and Lévy (56) attempted to assay choline by finding the greatest dilution of the unknown solution at which a special iodine reagent under specified conditions would just give the characteristic periodide crystals. In their procedure the limiting choline concentration is about 1:40,000 to 1:50,000. Neurine and tetramine give a similar reaction at even greater dilution, trimethylamine at 1:2,500, trimethylamineoxide at 1:1,000 and betaine at 1:500.

Carayon-Gentil (65) (66) claims that Florence's reagent does not precipitate all the choline in extracts of insects and marine invertebrates and reports finding that peptides interfere with the precipitation.

The chemical composition of the choline periodide precipitate, the conditions under which it forms and its suitability for use in the quantitative estimation of choline, were studied by Staněk (67). He found that a crystalline ennea-iodide or an oily hexa-iodide may form, depending upon the conditions. Both forms of the periodide are very insoluble in water. Staněk proposed, therefore, the use of potassium tri-iodide as a reagent for the quantitative precipitation of choline and reported recoveries of 96–101 per cent when analyzing known solutions. He described how choline may be recovered from this precipitate by treatment with finely divided copper and cupric chloride. After filtering off the insoluble cuprous halides, traces of copper are removed from the filtrate with hydrogen sulfide and upon again filtering and evaporating, choline chloride is obtained.

Staněk (68) soon discovered that betaine also is precipitated by potassium tri-iodide, but that this occurs only in acid solution. Since choline is precipitated in neutral, or even weakly alkaline solution (bicarbonate or borate), the reagent may be used for the separation of the compounds as well as their individual determination. The procedure separates choline and betaine quantitatively from inorganic salts and many other types of compounds found in plant extracts and avoids the use of phosphotungstic acid and mercuric chloride.

Staněk (68) found many other basic substances to be precipitated with the tri-iodide reagent. In the paper in which he gives final details of his method, Staněk (69) states that proteins, peptones, purines, alkaloids, trimethylamine, trigonelline, stachydrine, and muscarine are also precipitated. A valuable critical study of the periodide procedure for the isolation and estimation of choline soon was reported by Kiesel (70) in which further interfering substances are mentioned.

Sharpe (71) devised a quantitative chemical method for the determination of choline which was based on Staněk's work. The periodide precipitate is washed free of excess reagent with ice-cold water and decomposed with dilute (1:1) nitric acid. The liberated iodine is extracted with chloroform, the latter is then washed with water to remove nitric acid and the iodine is titrated with 0.05 *N* sodium thiosulfate. Sharpe recovered 90.6 per cent, 91.5 per cent, and 94 per cent, respectively of choline added in amounts of 17.0 mg., 4.9 mg., and 1.9 mg. to 20 to 50 cc. portions of blood. However, incomplete working directions made it difficult for others to repeat the procedure exactly, and it did not receive wide acceptance.

Roman (72) described the first micro-chemical method for the estimation of choline. A similar procedure was proposed (but not described in detail) within a few months and apparently independently by Maxim (73). Roman reinvestigated the Staněk method very carefully and showed that for a micro-procedure it is necessary to have the iodine concentration as high as possible while keeping the potassium iodide concentration as low as possible. However, too great a concentration of iodine must be avoided lest it be precipitated upon dilution of the reagent in the test solution, for it is difficult to wash the choline precipitate free of iodine without loss of some periodide. Roman believed the conditions were adjusted optimally if 0.3 cc. of a reagent containing 157 g. iodine and 200 g. potassium iodide per liter were used per cc. of choline solution. Quantities of choline up to 5 mg. per cc. can be handled by the reagent. Smaller quantities (down to 0.005 mg.) can be determined with equal accuracy (average errors ±1 per cent, maximum errors 5 per cent) but require the same amount of reagent to maintain a suitable medium for maximum precipitation. For details of the procedure the original paper should be consulted. Several modifications of Roman's method have been published recently (74) (75).

Erickson, Avrin, Teague, and Williams (74) improved the periodide micro-method by suggesting the use of an alundum filter stick, of the immersion type, which enables one to remove the supernatant fluid (while the centrifuge tube containing it remains in the ice-bath) without disturbing the precipitate or allowing it to warm up and lose iodine. A further improvement was the introduction of a bromine oxidation to convert the iodine to iodate; the latter is then allowed to oxidize added iodide, liberating six atoms of iodine per atom in the original ennea-iodide precipitate. Thus one obtains 54 titratable atoms of iodine per mole of choline precipitated. They use 0.005 N thiosulfate for the final titration, 1.00 cc. being then equivalent to 0.01122 mg. choline. Details given in the original paper indicate that the procedure is not difficult to follow. It appears to be a valuable contribution to the still imperfectly investigated subject of the micro-determination of choline in biological materials.

At the present time the most popular micro-method for the determination of choline appears to be by precipitation as reineckate. Quaternary ammonium bases form much less soluble reineckates than do tertiary amines, which in turn are less soluble than those derived from secondary amines. Heterocyclic compounds also form insoluble products, and hence many alkaloids are precipitated by ammonium reineckate. Further, the ω-amino acids form sparingly soluble reineckates (difference from α-amino acids). It is obvious, since so many different types of compounds are precipitated by ammonium reineckate, that it is by no means a specific reagent for choline and/or acetylcholine (76).

When Paal (77) used ammonium reineckate to determine the amount of choline liberated during hydrolysis of lecithin, the absence of related quaternary ammonium bases permitted use of the reagent. However, addition of reinecke salt or the free acid to blood filtrates and organ extracts, as practised by Kapfhammer and Bischoff (78) and Beattie (57), is an entirely different matter. Bischoff, Grab, and Kapfhammer (79) reported finding, with their reineckate procedure, up to 194 γ acetylcholine per gram in skeletal muscle, while by bio-assay Plattner and Krannich (80) and Chang and Gaddum (81) could not demonstrate more than 0.08 γ per gram, a difference of almost 2500-fold. The former workers also reported finding up to 45 mg. per kg. of free choline (as chloride) in muscle.

During the period 1933 to 1935, Strack and his collaborators in Leipzig partially clarified the situation as far as the determination of free choline is concerned. They showed (82) that certain tissues, notably liver and placenta, possess enzymes which liberate choline rapidly from phosphatides, and that the alcohol extraction procedure of Bischoff, Grab, and Kapfhammer does not inhibit the enzymes rapidly enough. However, Strack, *et al.* (83) could not find any free choline in beef, dog, or rabbit muscle, even after 5 hours incubation, so that liberation from complexes could not account for the considerable quantities apparently isolated from muscle by Bischoff, *et al.* Strack, *et al.* used a hot water extract which was evaporated to dryness, taken up in alcohol and precipitated with mercuric chloride. The precipitate was treated with hydrogen sulfide and the solution from this, after concentration, was treated with gold chloride. No chloroaurate of choline was obtained from skeletal muscle. However, when a reineckate precipitation was used, an insoluble product similar to that of Bischoff, *et al.* was obtained from which a chloroaurate could be prepared which resembled that of choline. On careful investigation it proved to be the chloroaurate of carnitine (m.p. 155°, Au = 39.36 per cent).

This observation led Strack and Schwaneberg (84) to study more carefully the reineckate procedure for the estimation of organic bases in tissues. In spite of its lack of specificity, they found ammonium reineckate to be a very useful reagent for the isolation of some of the bases. The separation of simpler bases depends upon careful adjustment of pH, but this is unnecessary with the quaternary ammonium bases. By extracting the precipitate with alkali the reineckates of compounds containing a free or potential carboxyl group may be dissolved. Choline reineckate, being insoluble in dilute ammonia, may thus be separated from the corresponding salts of betaine and carnitine. This treatment is claimed to effect a better separation of choline and betaine, for example, than does Staněk's periodide procedure.

A colorimetric modification of the reineckate method for the estimation of choline and acetylcholine was proposed by Beattie (57). The precipitate is dissolved in acetone giving a pink to bright red solution, the color of which is compared with that of a standard acetone solution of the appropriate reineckate or with that of an artificial standard (methyl red) solution. Values determined in this way on extracts of natural products are certain to be too large since other substances are also carried down as reineckates.

The reineckate procedure of Jacobi, Baumann, and Meek (85) is undoubtedly somewhat more specific. The ground tissue is extracted with boiling 1:1 alcohol-ether mixture for 3 minutes. The filtrate is taken almost to dryness on a steambath and then saponified for 2 hours at 80° with baryta. This not only liberates choline from its complexes but probably also destroys some of the interfering substances. After neutralization with acetic acid and filtration, the choline is precipitated with ammonium reineckate. The mixture is allowed to stand in the cold for 12 hours to ensure complete precipitation. After filtration the choline reineckate is dissolved in acetone and the color intensity is measured with an Evelyn photoelectric colorimeter, using a light filter which transmits at 520 mμ, or a spectrophotometer may be used. The chemical results were found to agree with bio-assays performed by the method of Chang and Gaddum (81), using the acetylation technique of Fletcher, Best, and Solandt (86).

Engel (87) has recently described improvements on this procedure. Much more exhaustive extraction is advised and methanol is recommended as a superior solvent. The combined extracts are taken to near dryness in vacuo and the residue is saponified with baryta for 2 hours at 100°. After neutralization the choline is precipitated with a methanol solution of reinecke salt, left for 4 hours at 3°, filtered and washed three times with small portions of cold ethanol (3°). The precipitate is then dissolved in acetone and the concentration of choline reineckate is determined with a photoelectric colorimeter, using filter 520. A biological assay is also proposed by Engel in which the protection afforded by the sample is compared with that of known amounts of choline in preventing kidney hemorrhages in rats on choline-deficient diets.

Shaw (88) attempted to devise a micro-procedure of greater specificity by performing the reineckate precipitation in 0.1 N sodium hydroxide solution. The small precipitate is suspended in sufficient water to give a turbidity equal to that of a standard tube. It is then placed in a water-bath at 60° for 2 minutes to dissolve the choline reineckate; any insoluble material is rejected. A special iodine solution is then added and the resulting brown color is compared with that of a choline standard treated similarly. Upon adding alcohol, the color due to choline is discharged. By difference, the color due to choline in the sample is obtained. For exact details the original must be consulted.

Lintzel and Fomin (89) have described a micro-method of estimation which depends upon the oxidative degradation of choline to trimethylamine under the influence of an excess of potassium permanganate added slowly to a hot, strongly alkaline solution. The volatile bases which distil from the reaction mixture are absorbed in hydrochloric acid. The acid contents of the receiver are evaporated to dryness, an excess of formaldehyde is added, the mixture is made strongly alkaline with caustic soda and the trimethylamine (the only base volatile from such a mixture) is aerated into standard sulfuric acid (0.02 N). The unused sulfuric acid is back-titrated with 0.02 N trimethylamine solution. Conditions were found which caused a quantitative decomposition of choline, yet which did not appreciably oxidize trimethylamine. Betaine and γ-butyrobetaine are claimed to be relatively stable under the same conditions, but neurine and carnitine behave as does choline; no data for other betaines or quaternary ammonium bases are given. Lintzel and Monasterio (90) simplified and shortened the procedure by eliminating the first acid absorption. The volatile bases are passed into an ice-cold mixture of formaldehyde and strong sodium hydroxide. Ammonia and mono- and dimethylamine are held back, only trimethylamine being carried over to the standard acid in the receiver. Although Lintzel and Monasterio devised the method for estimating lecithin in blood and plasma, it can be used to estimate choline in any mixture provided other quaternary ammonium bases are known to be absent. The only weakness of this ingenious and simple method is its lack of specificity, although it is probably at least as specific as many other procedures which have been proposed for the estimation of choline.

The quantitative method proposed by Klein and Linser (91), based on a simple alkaline degradation of choline to trimethylamine, naturally suffers from the same lack of specificity as does Kauffmann's test. However, it is useful when dealing with somewhat purified material, such as a lecithin-rich fraction. Various problems associated with the estimation of free choline (particularly the difficulty of effecting a clear-cut separation of choline from phosphatides) are discussed by Klein and Linser.

Most of the recently devised micro-methods appear to be satisfactory for use upon hydrolyzed phospholipid materials. In other words, they may be used to determine total choline in a specially purified fraction from which other quaternary ammonium bases are absent. However, a chemical method suitable for the determination of free choline in body fluids or tissue extracts has not yet been described. The failure to discover a precipitant or chromogenic compound with high specificity for choline leaves the chemical estimation of this important metabolite in tissues as a problem for the future.

The bio-assay is still the most specific and sensitive method for detecting choline and of estimating small amounts with approximate accuracy, but it possesses many dangerous pitfalls for the chemist and is only reliable in the hands of a competent physiologist. Alcohol, calcium, potassium, histamine, adenosine compounds, traces of protein, lecithin, cholesterol, and several as yet unidentified compounds occurring in tissue extracts, may cause profound disturbances in the assay. The method was proposed in 1906 by Hunt and Taveau (92) who observed that acetylcholine possesses from 1000 to 100,000 times the physiological activity of an equivalent amount of choline, depending upon the organ or tissue preparation used in the assay. For further discussion of the pertinent literature and for details of the method one may consult Chang and Gaddum (81) and Fletcher, Best, and Solandt (86).

V. Choline as a Dietary Factor

This aspect of the choline investigations was a direct outgrowth of the discovery of insulin. Banting and Best were not particularly interested during the early phases of the insulin work, in long-continued experiments on diabetic animals. They felt that it was sufficient, at the time, to show that all signs of the disease could be eliminated for six or seven weeks by the administration of the anti-diabetic hormone and that the abnormalities reappeared promptly upon cessation of the injections. In the light of our present knowledge it was fortunate that when Professor Macleod and his group initiated the studies, in which they observed insulin-treated depancreatized animals for several years, that an adequate diet was not always used. A great deal of knowledge of the accessory food factors and of other aspects of nutrition has, of course, accumulated since that time. The depancreatized dog suffers from the loss of several of the digestive ferments and the absorption of food material from the intestine is seriously impaired. When Allan, Bowie, Macleod, and Robinson (93) in Toronto, and Fisher in Chicago (94) noted large yellow livers in the insulin-treated animals, many possible explanations suggested themselves. When it was found that the abnormal conditions could be prevented by the inclusion of raw beef pancreas in the diet, it became apparent that some constituent of this tissue was responsible for the improvement. Dr. Hershey (95) first alone and later with Dr. S. Soskin (96) working in Professor Macleod's laboratory, studied the effects of feeding crude egg-yolk "lecithin" to the diabetic dogs receiving the diet which favored the production of fatty livers. This work was in progress when one of us (C. H. B.) succeeded Professor Macleod as Professor of Physiology in Toronto. Macleod was not, at that time, convinced that a definite result attributable to the "lecithin" had been obtained. It became apparent, however, as one watched the subsequent experiments, that the phospholipid mixture was effective in a manner similar to that of pancreas. The logical inference was, therefore, that the phospholipid fraction or some unidentified substance associated with this material in the pancreas was responsible in part at least for the effect on liver fat.

Experiments on depancreatized dogs are extremely tedious, and in studies of this type, unless a large number of animals are observed, the results are difficult to interpret. Huntsman, Hershey, and Best (97) decided to make studies on the white rat in an effort to enlarge knowledge of the factors which control fat deposition in the liver. They investigated the effect of, first, a crude and then a highly purified lecithin on the fatty livers which they found could be produced in the rats by feeding a diet very rich in fat. Purified lecithin turned out to be effective in the prevention of fatty livers and sometime later, it was established that the active constituent of the lecithin was choline (98). The addition of pure choline to the diet prevented the deposition of excess fat in the liver.

Later it was found that as little as 1 mg. per rat per day is an effective dose. Best, Ferguson, and Hershey (99) showed that choline, when given in sufficient amounts to diabetic dogs, prevented the development of fatty livers and alleviated the condition when it was administered in curative experiments.

This conclusion has been supported by all subsequent investigators (100) (101) but it has been shown by Dragstedt and his colleagues (100)

(102) that other factors contribute to the effect of the pancreas. This aspect of the subject will be referred to again later.

The discovery of this effect of pure choline focussed attention on the food materials which can be shown to contain this substance, and a diet which contained much less choline than the mixed ration which previously had been provided was devised. This finding permitted a more dramatic demonstration of the effect of choline on liver fat in both rats and depancreatized dogs.

In subsequent experiments (103) (104) it was shown that the feeding of choline prevented the deposition of neutral fat and to a lesser extent that of the cholesterol esters in the livers of animals receiving pure cholesterol in their diets. It thus appeared that choline was involved in the metabolism of cholesterol as well as in that of neutral fat. In curative experiments choline accelerated the removal of cholesterol esters as well as neutral fat from the liver.

Further details of those and other aspects of the early choline studies will be found in the reviews by Best and Ridout (105), Frame (106), Griffith (107) and in the symposium on the biochemistry of choline (108). Certain phases of this subject have recently developed with great rapidity and most of the space available will therefore be devoted to them.

VI. The Lipotropic Effect of Proteins and Amino Acids

The first intimation that choline and betaine were not the only substances possessing lipotropic activity came in 1935 when Best and Huntsman (109) noted that casein exerted a similar influence. The observation was made during a study of the curative action of choline on the fatty liver of rats in various states of nutrition. In one of their experiments, in order to obtain a diet absolutely free of choline, they fed only sucrose to rats with fatty livers and noted that the livers became even more fatty. Upon repeating this experiment with 20 per cent casein replacing an equivalent amount of sugar, the further increase in liver fat was not observed. This first evidence of the lipotropic effect of casein was communicated, before publication, to Channon, who, with his collaborators in Liverpool, subsequently confirmed and extended the results (110). Rats were fed a low choline, high fat (40 per cent) diet supplemented with increasing amounts of casein up to 50 per cent. After three weeks the analyses showed that the amount of fat in the liver depended on the quantity of protein in the diet and that the liver fat decreased with increasing amounts of dietary protein. This effect of casein was also demonstrated on the glyceride fraction of the cholesterol fatty liver (111).

In curative experiments Best and Ridout (112) demonstrated that the cholesterol fraction of liver lipids was definitely decreased by a high casein diet. The experimental animals received a small daily dose of cholesterol (18 mg.). Beeston, Channon, and Wilkinson (111) in preventive experiments, failed to demonstrate an effect of casein on the deposition of cholesterol esters. These investigators fed, however, about ten times as much cholesterol (2 per cent of the diet).

Best, Grant, and Ridout (113) found that under certain experimental conditions a maximal lipotropic effect of casein was obtained at a 30 per cent level in the diet. They showed that gelatin possessed little or no lipotropic activity but that carefully prepared dried egg white and beef muscle powder were effective. The former had approximately the same potency as casein, the latter definitely less.

In 1936, Beeston and Channon (114) in confirmation of Curtis and Newburgh (115) showed that cystine caused a large increase in the liver fat of rats on a low casein, high fat diet. Above a certain low level of cystine intake the deposition of fat does not bear any definite relationship to the amount of cystine ingested. This observation probably has a bearing on the failure of added cystine to influence the liver fat of rats on certain diets in which the basal protein probably provided sufficient cystine to produce a maximum effect.

Preliminary studies of Beeston and Channon (114) failed to reveal an amino acid in casein with lipotropic properties, but showed that lysine, glutamic acid, aspartic acid, serine, glycine, and phenylalanine lacked activity in this respect when tested on the fatty liver produced by an alipotropic diet. In 1937, Beeston, Channon, and Platt (116) reported that cystine also caused a considerable increase in the glyceride fraction of the cholesterol fatty liver. Its effect was counteracted when the dietary casein was increased to 15–20 per cent. They further noted that glycine, when present to an extent greater than 1 per cent in diets producing cholesterol fatty livers, progressively increased the liver glyceride. No further mention has been made of this and no confirmation has yet been noted. In the same paper they reported that experiments both on the fatty liver and the cholesterol fatty liver suggest that tyrosine has some effect in preventing glyceride deposition. This lipotropic action of tyrosine is reaffirmed in a later paper (117), in which they also report having retested and found negative, serine, lysine, aspartic acid, and phenylalanine. Negative results were also reported with alanine, proline, hydroxyproline, histidine, valine, leucine, and arginine. In 1941, Singal and Eckstein (118) confirmed the negative effects of *dl*-valine and *dl*-leucine. They also found that djenkolic acid and *dl*-isoleucine possessed no lipotropic activity. The same workers (119) using mice, showed definitely that cysteine and homocystine act in the same manner as cystine on liver fat. This antilipotropic effect of homocystine had been indicated earlier by Channon, *et al.* (120) who reported that further evidence was necessary to confirm the point.

A great step forward was made in 1937 when Tucker and Eckstein (121) confirmed the effect of cystine on liver fat and made the interesting discovery that methionine was lipotropic and probably responsible in large part for this action of casein. Their results indicated that the effect of methionine was more easily seen if the basal diets were such that the liver fat in the control rats was high. They suggested that the lipotropic action of casein—and presumably any protein—was due to "a resultant effect of the simultaneous opposing influences of the cystine and methionine of the diet." The lipotropic action of methionine has since been repeatedly confirmed (122) (123).

Channon, Manifold, and Platt (122) showed that methionine exerted its lipotropic effect on the glyceride and cholesterol fractions of the cholesterol fatty liver. They also found that the effect of the methionine was more marked when egg albumin was used as the basal protein. They attributed this finding to the fact that the liver fat was much higher in the albumin controls than in the casein controls. Some of their earlier experiments had shown that a level of 0.5 per cent dietary methionine exerted little lipotropic effect when the liver fat of the controls was only moderately high (16 per cent).

Channon, *et al.* (124) investigated the lipotropic effect of a series of proteins and noted marked differences in them. A short time later, Tucker and Eckstein referring to this paper, pointed out that the lipotropic activities corresponded roughly with the methionine content of the proteins.

The first attempts to assess the effects of cystine and methionine on liver fat gave apparently anomalous results. Tucker and Eckstein (125) showed that the addition of cystine to a diet containing 40 per cent lard and 5 per cent gliadin exerted little effect, although when casein was used as the basal protein the effect was marked (121). When albumin was used as the basal protein a similar inability to demonstrate the cystine effect was encountered (126). The probable explanation is found in the results of Beeston and Channon (114) who showed that, under their experimental conditions the effect of cystine in increasing liver fat was not proportional to the amount fed when more than 7 mg./rat/day was provided. Basal diets containing 5 per cent gliadin or albumin contain more than this amount of cystine in the average daily ration of a rat weighing 150–200 g.

In 1939, Singal and Eckstein (119) reported that casein exerted the same lipotropic effect in mice as had been observed in rats. They found, however, that arachin failed to influence the fatty livers of mice and attributed this to its low methionine content. Some support for their explanation of the non-lipotropic action of arachin is found in the observation of Best, Grant, and Ridout (113), published some years previously, that gelatin (which contains little or no methionine) possesses no significant lipotropic activity.

The next important advance was the suggestion of du Vigneaud, *et al.* (127) that methionine exerts its lipotropic action by contributing its methyl group for the synthesis of choline, a transmethylation reaction which was subsequently proven to occur (128). This phase of the methionine-choline relationship is discussed elsewhere in the review.

In 1940, Best and Ridout (123) found that the lipotropic activities of both the *d*- and *l*-forms of methionine were of the same order. They reported further that when the methionine in the diet was increased over 0.5 per cent no corresponding decrease in liver fat was observed although there was still a considerable amount of fat in the liver (10–17 per cent). They studied the relative lipotropic effects of (a) 30 per cent casein, and (b) cystine and methionine as free amino acids in amounts equivalent to the quantities in this dietary level of casein. The average liver fat value from the methionine-cystine supplemented diet was 16.8 per cent and from the casein supplemented diet 7.1 per cent. They were led to the conclusion, already suggested by Tucker and Eckstein (125), and later by Channon, *et al.* (126), that there were probably other factors in addition to cystine and methionine which may be involved in the explanation of the lipotropic effect of proteins.

Channon, *et al.* (126) confirmed Best and Ridout's finding concerning the limited lipotropic action of methionine. They pointed out that when an 8 per cent casein diet was supplemented with 0.15–0.2 per cent methionine (0.42–0.47 per cent in all) the maximum methionine effect was obtained; whereas casein exerts an increasing lipotropic effect up to 30 per cent which is equivalent to a total of 0.83 per cent methionine in the diet. They conclude that either methionine exerts its effect quite independently of choline or some factor other than the provision of labile methyl groups becomes limiting for the body synthesis of choline. They mention in this connection the possibility—already suggested by du Vigneaud, *et al.* (127)—that the production of aminoethanol might be the limiting factor. It is interesting to note that a short time later DeWitt Stetten, Jr. (129) demonstrated that ethanolamine serves as a precursor for the biological synthesis of choline.

Tucker, Treadwell, and Eckstein (130) found that 5 per cent casein diets supplemented with enough cystine and methionine to bring the level of these two amino acids up to that contained in 15 and 20 per cent casein diets, gave lower liver fat values in rats than did the high protein diets. This finding is apparently in direct conflict with that of Best and Ridout (123) mentioned above. It should be pointed out, however, that the latter workers were experimenting at a much higher level of dietary protein—35 per cent instead of 15 and 20 per cent—and that in other experiments they used 5 per cent beef powder protein in the basal diet in place of casein. Provided that the beef powder used by Best and Ridout did not exert any influence other than that which may be attributed to its methionine and cystine content (3.19 and 0.97 per cent respectively) then these combined results indicate that between the 20 and 35 per cent protein levels the lipotropic effect of the dietary protein increases steadily whereas that due to a 5 per cent basal protein diet supplemented with increasing amounts of methionine rapidly reaches a maximum. The reason for this phenomenon may be as Channon, *et al.* (126) have expressed it that "added methionine is incapable of exerting its full effect in the absence of some other protein constituent." On the other hand the deficiency may be more general and involve all the essential amino acids and the nitrogenous equilibrium of the animals.

These divergent findings prompted Treadwell, Groothuis, and Eckstein (131) to reinvestigate the question of the relative lipotropic efficacy of 15 and 20 per cent protein diets as compared with 5 per cent protein diets supplemented with sufficient methionine and cystine to raise the quantities of these sulfur-containing amino acids to the same levels contained in the high casein rations. They confirmed their previous results (130) and pointed out that there was no evidence whatsoever in their results to confirm the previously quoted contention of Channon, *et al.* (126). On the contrary the free amino acid appeared to be superior to the same amount found in the protein. It should be noted that neither group of workers has attemnted to repeat exactly the other's procedure. Treadwell, *et al.* call attention to the fact that their rats, which weighed 100–125 g. ate much more and gained more weight on the high protein diet than on the methionine-cystine diets. They advance the theory that since there was much more new tissue protein laid down in the rats on the high protein diets that this involved an increased demand for methionine and because of this demand less methionine may have been available for lipotropic action. This is in effect the same idea expressed by and demonstrated by Griffith and Mulford (132) and it is therefore evident that the difference in food intake alone might be held responsible for the different lipotropic effects of the two diets. They conclude that it would be of interest to repeat this work on adult rats in which the growth factor is practically ruled out. A better method of eliminating the ambiguity caused by the excessive food intake of the one group would be to use the paired feeding technique.

One of Treadwell, *et al.*'s experiments involved the addition of cystine in increasing amounts to a low casein (5 per cent), high lard (40 per cent) basal diet supplemented with sufficient methionine to make the total methionine content equivalent to that of a ration containing 20 per cent casein as the sole source of protein. Contrary to the results reported by Beeston and Channon (114) a definite increase in liver fat was observed when the cystine level was raised. Although it is not pointed out, this finding indicates that under the conditions used by these workers, there appears to be an antagonism between the effects of the two amino acids even when fed at high levels. The converse experiment in which increasing amounts of methionine were added to the basal diet containing 0.6 per cent cystine, showed that methionine exerted an additional lipotropic effect when fed in excess of 0.5 per cent. It should be recalled that under slightly different conditions Best and Ridout (123) and Channon, *et al.* (126) found that increasing the methionine content of the diet above 0.5 per cent did not produce any further effect on the liver fat.

The fact that food intake has a marked effect on liver fat—at least in young rats—has been demonstrated by Griffith and Mulford (132). They showed that with increasing intake of food the liver fat increased. In addition, Mulford and Griffith (133) have pointed out that the usual low protein diets (18 per cent) used in producing renal lesions and fatty livers in young rats, contain a sub-optimal level of dietary sulfur, 0.14 instead of 0.19 per cent, and that a supplement of 0.05 per cent of extra sulfur as cystine results in longer and heavier animals, these gains being obtained with little or no increase in food consumption. It therefore appears that cystine brings about an increased utilization of food under the conditions described, and they advance the theory that cystine exerts its anti-lipotropic effect by virtue of raising the metabolic level nearer to normal thus creating an increased demand for lipotropic factors. These points will be discussed further in the section on intermediary metabolism of choline and other lipotropic factors.

The seemingly conflicting nature of some of the mass of experimental data presented to date on the lipotropic effect of the proteins makes the task of drawing any clear cut conclusions a difficult one. However, the following points may be noted.

Cystine is anti-lipotropic but this action is probably non-specific and due to the raising of the metabolism nearer to the normal level thus resulting in an increased demand for lipotropic factors. Attention must be drawn, on the other hand, to the work of Treadwell, *et al.* (131) whose results indicate the possibility of a direct antagonism between methionine and cystine.

Methionine exerts a lipotropic effect through its contribution of labile methyl groups for the synthesis of choline. On low protein diets, high levels of methionine may not exert their full effect because of a deficiency of some other protein constituent. This might explain why 5 per cent basal protein diets supplemented with the same amounts of methionine and cystine contained in a 30 per cent casein diet fail to lower the liver fat to the same extent as the casein itself does. It should be borne in mind that even in the experiments of Treadwell, Groothuis, and Eckstein the liver fat is never reduced below 10 per cent by methionine alone or in combination with cystine. Adequate amounts of casein will produce much lower values.

VII. INTERMEDIARY METABOLISM OF CHOLINE AND OTHER LIPOTROPIC FACTORS

The demonstration in 1938 that methionine is an indispensible amino acid (134) and the removal of cystine from this category, evoked a renewed interest in the rôles of the S-containing amino acids in nutrition. Butz and du Vigneaud (135) had previously discovered that methionine loses its methyl group when boiled with strong sulfuric acid and from the reaction mixture a new S-containing amino acid, homocystine, was isolated. Although to date neither homocystine nor homocysteine has been isolated from food or tissue proteins the above observations naturally raised the question as to whether methionine loses a methyl group in intermediary metabolism and as to the ability of homocystine to replace methionine in the diet.

Both du Vigneaud and Rose commenced studies along these lines in 1938. The methionine-free diets used in New York and Urbana were superficially identical, but the two groups obtained strikingly different results—the rats in du Vigneaud's laboratory not only failed to grow but lost weight. Rose's animals grew, but at a subnormal rate (136) (137). The discrepancy was eventually traced to the vitamin B supplements chosen by the two groups. Rose and Rice used a mixture of tiki-tiki and a milk vitamin concentrate; du Vigneaud had used crystalline thiamin chloride, riboflavin, and nicotinic acid (0.02 mg. each daily) with 25 mg. of ryzamin-B. When the rats in du Vigneaud's laboratory were autopsied at the end of the experimental period it was discovered that they had not only failed to grow but that the livers appeared fatty. This observation suggested the possibility of a choline-deficiency in du Vigneaud's diet and this was soon proven to be the case. The crude vitamin preparations used by Rose were subsequently shown by du Vigneaud, *et al.* (138) to contain considerable choline—about 3.5 mg. in the daily supplement, or ten times that found in the daily dose of ryzamin-B which had been used by du Vigneaud's collaborators.

At the Toronto meeting of the American Society of Biological Chemists in 1939, du Vigneaud, Chandler, Moyer, and Keppel (139) first reported that rats were unable to grow on a synthetic diet which contained homocystine or homocysteine in place of methionine, but that when choline was added growth promptly ensued. Their subsequent publication (138) of the details of the work proved to be the first chapter in a rapidly growing volume of nutritional research which led to a new metabolic chemical change being discovered and definitely established—transmethylation. The interrelationships between choline and other methylated compounds have been considerably clarified during the last three years, particularly so during the past few months. An excellent review of the subject by du Vigneaud (140) describes the progress of these studies to the end of 1941.

The fact that homocystine cannot replace methionine in the diet, except in the presence of choline (or related substances), was soon confirmed in other laboratories (141) (142). The explanation proposed by du Vigneaud, *et al.* (138) was that choline acts as a donor of methyl groups in the synthesis of methionine from homocystine. By visualizing the process as a reversible one, they were led to suggest that dietary methionine (which is lipotropic) may be a precursor of choline so far as methyl groups are concerned. The hypothesis was advanced that methyl groups in a utilizable form are indispensible in the diet because the animal organism is incapable of generating methyl groups for the essential methylations. The idea of transmethylation reactions had been in mind for several years. As early as 1935, both Lewis (143) and Brand, *et al.* (144) had raised the question as to whether the methyl group believed to be released during metabolism of methionine was utilized in the synthesis of creatine, but at that time no technique was available for settling the point unequivocally. The hypothesis suggested by du Vigneaud, *et al.* to account for the choline-homocystine-methionine relationship was quickly subjected to critical test, utilizing tracer elements (deuterium, radio-active sulfur, and heavy nitrogen) in the dietary components and following them in compounds isolated from the tissues and excreta.

The close chemical relationship of betaine to choline, and the known lipotropic properties of betaine, led Chandler and du Vigneaud (145) to test its ability to induce growth in diets containing homocystine but no methionine. It proved to be active but not as potent as choline and it was tentatively assumed that it exerted its effect through conversion to choline in the body. The work of Stetten (146) (147) indicates that only the methyl groups of betaine are utilized in choline synthesis (see also page 29).

Whether the effect of choline on growth might be an indirect one, by preventing the development of fatty livers in animals on diets containing homocystine as the sole source of sulfur-containing amino acids, was examined by Moyer and du Vigneaud (148); for it appeared possible that choline, by preventing liver damage, might allow that organ to carry on methylations or other reactions essential to growth. When triethylcholine, which had already been shown to be lipotropic (149), was fed to animals on diets containing homocystine in place of methionine, development of fatty livers was prevented but no growth occurred. Obviously, the presence of a fatty liver was not entirely to blame for failure of growth in the experiments in which choline was not fed.

The proof of the reality of the transmethylation reactions which had been postulated was brought out in a series of papers from du Vigneaud's laboratory describing the results of feeding compounds containing labelled methyl groups. By catalytic reduction of carbon monoxide with heavy hydrogen, deuteriomethyl alcohol was obtained which was converted to deuteriomethyl iodide. The latter compound was used to methylate sodium homocysteinate in liquid ammonia, giving deuterio-methionine, and to methylate ethanolamine, giving deuteriocholine.

When deuteriomethionine was fed at a 70 mg. level for 3 weeks to rats on a diet lacking choline and methionine (150) the choline subsequently isolated from the carcasses as chloroplatinate was found to contain deuterium. That all of the deuterium was present in the methyl groups of the isolated choline was later demonstrated by oxidative degradation with alkaline permanganate (151). The trimethylamine thus liberated was distilled over into hydrochloric acid and isolated as chloroplatinate. This material contained all the deuterium originally present in the choline, thus clearly establishing the biological transfer of the intact methyl group from the methionine into the choline molecule.

It was simultaneously shown that labile methyl groups from methionine play other rôles in metabolism. The muscle creatine of the rats fed deuteriomethionine (isolated as the zinc chloride complex of creatinine) was shown to contain deuterium and it was demonstrated that the deuterium was in a deuteriomethyl group. This was established by hydrolyzing the creatinine to sarcosine with baryta and decomposition of the latter to methylamine with silver oxide. The methylamine was separated as the chloroplatinate and found to contain all the deuterium atoms originally present in the creatine, again showing that the deuteriomethyl group of the dietary methionine is transferred intact, this time into the creatine molecule. As might be expected from the above findings, the creatinine excreted in the urine was also found to contain the deuteriomethyl group, and the deuterium content of the methyl groups of the choline, creatine, and creatinine were found to be of similar magnitude.

In subsequent feeding experiments they took advantage of the latter finding to follow the degree of replacement of normal methyl groups in body compounds by deuteriomethyl groups, thus enabling them to avoid premature sacrificing of the animals and also to avoid wasting the costly nutriments in unnecessarily prolonged periods of feeding.

Deuteriocholine was fed to rats on a diet lacking methionine and choline, but containing homocystine, and after three weeks the animals were sacrificed. The deuterium content of the tissue creatine showed that in that time 24 per cent of the methyl group had been replaced by that coming from the deuteriocholine. After eight weeks on the diet, 29 per cent of the theoretical maximum deuterium content had been attained. Deuterium was found in the methionine from tissues of rats on a similar diet. This evidence that the methyl groups of choline and methionine can be interchanged and also utilized in the synthesis of creatine established beyond doubt the labile nature of these *N*-methyl and *S*-methyl groups, thus giving direct and incontrovertible proof of the occurrence of biological transmethylations.

Methionine has recently been shown to be more effective than choline (roughly ten times) as a methyl donor for creatine formation. Also, by feeding deuteriomethionine at 0.6 per cent and 1.4 per cent levels in the diet, the rate of methyl transfer has been found to be proportional to the amount fed (140).

The diets originally employed for the study of methyl transfer contained homocystine but no methionine. It has since been demonstrated by du Vigneaud, Simmonds, and Cohn, quoted in (140), that the methyl group of dietary choline appears in tissue creatine even if sufficient methionine and no homocystine are fed, although the amount of deuteriomethyl group present in the creatine is less on a diet of deuteriocholine plus methionine than on one containing deuteriocholine plus homocystine. This obviously indicates preferential use of methyl groups from methionine. By feeding 1.4 per cent deuteriomethionine in the presence of 25 mg. choline per day, transfer of methyl groups to newly formed choline still occurred. The presence of choline did not materially alter the amount of methyl transfer from deuteriomethionine to creatine, indicating that the methyl group of choline is either not directly transferred to creatine or undergoes transfer much less readily than does that of methionine. These *in vivo* findings agree with the results of *in vitro* experiments performed by Borsook and Dubnoff (152), who found that liver slices synthesize extra creatine from guanidoacetic acid in the presence of methionine but not in the presence of added choline.

Beeston and Channon (153) noted that addition of cystine to low-protein, low-choline diets increased the liver fat and the need of choline, an effect just the opposite to that subsequently found to be produced by the other sulfur-containing amino acid, methionine (154). The view held by some workers that there is a real antagonistic relationship between cystine and choline has been disputed by Griffith (155) (156), whose explanation receives support in the finding of du Vigneaud (140) that the feeding of cystine does not interfere with transmethylation reactions. Further confirmation comes from the recent report of Stetten and Grail (157) that the phospholipids of fatty livers caused by cystine feeding differ in composition from those due to choline deficiency: the former leads to deposition of extra lecithin, the latter to loss of lecithin. Griffith bases his contention on three clearly established facts: (1) the choline requirement is markedly influenced by the nutritional level; (2) the choline requirement is not directly related to the amount of cystine in the diet; and (3) the 18 per cent casein diets used in many of his pertinent studies is a sub-optimal source of cystine and of total sulfur, supplying only 0.14 per cent of the latter, whereas with adequate dietary choline 0.19 per cent of protein-sulfur appears necessary for normal growth of young rats. Griffith contends that supplementary cystine improves the nutritional level, increasing the demand for choline. That the effect of the added cystine is to improve the metabolic efficiency seems clear since gains in body weight and length are observed with little or no increase in the food consumption. Griffith has found that during the growth period, between the ages 25–45 days, young rats exhibit the greatest need for cystine (this may be supplied as such or as methionine, which appears to be used as cystine). The cystine supplement to an 18 per cent casein diet which produces the greatest stimulation of growth and therefore presumably maximum increase in efficiency of food utilization, is approximately the same as that which causes the greatest deposition of liver fat. Increasing the cystine beyond this point, even twenty-fold, causes no appreciable augmentation of liver fat. This work of Griffith, supported by data from other laboratories (even if the conclusions drawn from them were different) provides strong support for his contention that the apparent direct antagonism of cystine and choline is really the manifestation of a greater need of choline at the improved nutritional level induced by an adequate supply of dietary cystine.

To get further information about the lipotropic properties of casein, Mulford and Griffith (156) fed high-casein diets to determine at what dietary level of the protein (and methionine) the necessity of a choline supplement would be obviated. About 30 per cent of casein appeared necessary. The conclusion was also drawn from these experiments that methionine which is used as a source of labile methyl groups is not available as a source of cystine sulfur. The correctness of this remains to be proven.

That the sulfur of ingested methionine can be translocated into tissue cystine has been shown by Tarver and Schmidt (158) who fed methionine, containing radio-active sulfur, to young rats and subsequently isolated crystalline radio-active cystine from the hydrolyzed hair. The nature of the intermediate steps in the transformation has recently been elucidated by du Vigneaud and Stetten and will be discussed later.

The lipotropic activity of betaine, originally demonstrated by Best and Huntsman (159) and repeatedly confirmed (160) (161) (145) (162) is claimed to be about one-third of that of choline, suggesting that only one methyl group is available for transmethylation. This indication that not all methyl groups in biologically important compounds are available for transmethylation is borne out by the behavior of creatine and sarcosine, neither of which is lipotropic. They are not methyl donors, *i.e.* do not permit *in vivo* formation of methionine from homocystine (163) and do not protect young rats from hemorrhagic degeneration of the kidneys (164). That demethylation of sarcosine (a compound in which two of the three methyl groups of betaine have been removed), does occur in rats and rabbits with formation of glycine has been established (165) (166), but the methyl group is apparently destroyed by oxidation. One must distinguish, therefore, between *demethylation* and *transmethylation.*

The lipotropic activity of glycine-betaine led to a study of other betaines and by now ten others have been investigated. Only alanine-betaine (160) and cystine-betaine (167) have exhibited lipotropic activity. Ergothionine (thiolhistidine-betaine) (168) (138), trigonelline (nicotinic acid-betaine) (148) and the betaines of glutamic acid (169), serine (170), threonine (170) and allothreonine (170) do not exert any lipotropic action. Stachydrine (proline-betaine) and β-alanine-betaine, which were tested only as methyl donors, were ineffective (148).

Moyer and du Vigneaud (148) have made a very comprehensive study of compounds which can act as methyl donors. Of particular interest were a number of homologues of choline which they prepared and examined; the only one found to support growth with homocystine as the dietary source of sulfur was dimethyl-ethyl-hydroxyethyl ammonium chloride, *i.e.*, choline with one of the methyl groups replaced by ethyl. This compound, which is lipotropic, also prevents kidney hemorrhages (169) and perosis and favors fowl growth (171). Moyer and du Vigneaud have brought the literature up to date in a most useful table which presents not only the effect of the various compounds on growth with homocystine but also the lipotropic and anti-perotic activity, the ability to prevent kidney hemorrhages and the effect on the growth of fowl. The most significant feature is the high degree of specificity of structure in relation to the ability to act as a methyl donor. Only five out of about thirty-five compounds which have been tested were found to support growth under the conditions of their experiments: choline, calcium phosphoryl-choline, dimethyl-ethyl-hydroxyethyl ammonium chloride, lecithin and betaine.

Out of about fifty compounds which have been tested for lipotropic action by various workers, nineteen have been reported to possess activity. In some cases the findings are contradictory, *e.g.* *S*-methylcysteine has been claimed to be inactive (172) yet Singal and Eckstein (167) found it to be lipotropic and claimed similar activity for dithiodiglycolic acid.

The lipotropic activity of some of these compounds is difficult to explain, particularly that of dithiodiglycolic acid, but they may exert their influence indirectly upon the labile methyl supply by varying the quantity of precursors or methyl acceptors.

Recent studies of cystathionine, *ll*-*S*-(β-amino-β-carboxyethyl)-homocysteine, and of the fate of dietary serine have materially contributed to clarification of the part played by homocysteine in metabolism. Although cystathionine does not support growth of rats in the absence of methionine and choline it can replace cystine in a diet low in methionine (173). Binkley, Anslow, and du Vigneaud (174) found that slices of rat liver or a saline extract of liver can split the thio-ether, liberating cysteine. The cleavage occurs more rapidly under anaerobic conditions, but considerable hydrogen sulfide is then also liberated. The latter decomposition is inhibited by the presence of sodium cyanide. Muscle and kidney are inactive, as is boiled liver. Binkley and du Vigneaud (175) have more recently shown that an enzyme present in rat liver effects the conversion, under anaerobic conditions, in the presence or absence of cyanide, of a mixture of serine and homocysteine into cysteine. Methionine is much less effective than homocysteine in serving as the sulfur-containing precursor. The unnatural enantiomorphs (*d*-homocysteine and *d*-serine) are not acted upon appreciably by the enzymes. These findings support the theory of Brand, *et al.* (176), rather than those of Nicolet (177), or Toennies (178) concerning the path by which dietary methionine becomes converted into tissue cystine, but as Binkley and du Vigneaud point out the whole story is not yet clear.

The importance of serine in this cycle in the intact animal has been shown by Stetten's (179) discovery that dietary serine containing heavy nitrogen (N^{15}) leads to the formation of tissue cystine rich in isotopic nitrogen. This indication that the nitrogen (and probably the carbon chain) of serine is utilized in building the cystine molecule in the rat enhances the significance of the findings of Binkley and du Vigneaud and strongly supports their hypothesis concerning the formation of cystine from methionine *via* homocysteine and serine.

VIII. The Vitamin B Complex and the Lipotropic Factors

In 1935, Best, Huntsman, McHenry, and Ridout (180) reported a favorable influence of choline on the gain in weight of young rats receiving a diet low in lipotropic factors and rich in fat. Subsequently McHenry and Gavin and their collaborators have made a number of extremely important additions to our knowledge of the choline field. In 1936 (181) McHenry reported a relationship between thiamin and choline in the production of fatty livers. In thiamin-deficient animals, when no thiamin is provided, fat does not accumulate in the liver in the absence of the dietary lipotropic factors unless liberal amounts of fat are used. With thiamin and without fat in the diet, fat is formed and is deposited in the liver. This fat is rich in glyceride and low in cholesterol and its deposition is prevented by the administration of small amounts of choline. Some of the members of the B-complex (riboflavin, pyridoxin) also increase the deposition of fat in the liver but not unless thiamin is also supplied (182). Engel's work (183) on calcium pantothenate provides further evidence. He states that the addition of calcium pantothenate to a synthetic diet containing thiamin, riboflavin, pyridoxin, and choline, caused a 100 per cent increase in liver fat. A lack of thiamin or riboflavin resulted in low levels of liver fat. The omission of pyridoxin had no effect until, at the end of a 7 week experimental period there was a marked increase in the liver fat. This increase which was observed while the food consumption was low, is difficult to explain.

In 1937, MacLean, Ridout, and Best (184) showed that choline caused a gain in weight of rats which had previously been on a choline-free diet and they suggested that the presence of this substance in the diet favored the normal distribution of fat in the liver and the body depots. In other words it prevented fatty accumulation in the liver and increased the deposition in the fat stores of the tissues.

The results of studies (185) using deuterio fatty acids deposited in the tissues of rats, showed that when these animals were placed on a diet low in lipotropic factors, the fat which accumulated in the liver did not contain the labelled fatty acids. Longenecker, Gavin, and McHenry (186) demonstrated that the fat deposited in the liver of animals on an alipotropic[2] diet was not due to an increased absorption of fat from the intestines but was in all probability produced by the formation of fat from carbohydrate. A study of the effect of thiamin upon the deposition of liver and body fats has been made by McHenry and his collaborators. It would appear probable that the liver fat which accumulates in the absence of choline, is due in large part to synthesis from carbohydrate sources. McHenry and his colleagues have made the further most interesting suggestion that thiamin is essential for this conversion (187). This may account for the source of the deuterium-free fat deposited in the rat livers in the experiments of Barrett, Best, and Ridout (185) mentioned above, for vitamin B_1 was present in the diets which they used.

In 1938, Halliday (188) reported that a diet deficient in vitamin B_6

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[2] See Section XIX, page 47.

(pyridoxin) caused the production of fatty livers. The administration of liver preparations containing pyridoxin decreased the fat content of the liver. McHenry and Gavin in 1938 (189) observed that the body fat of rats was increased out of proportion to the food intake by supplementation of a diet containing thiamin and riboflavin with a source of vitamin B_6. Later these workers (182) using crystalline pyridoxin in conjunction with thiamin, riboflavin, and choline, showed that the pyridoxin caused an increase in body fat in the rats which were maintained on a fat-free diet. This effect was slightly augmented by the further addition of nicotinic acid. In 1941 (190) McHenry and Gavin presented evidence that pyridoxin was concerned in the conversion of protein to fat. They had previously shown that thiamin alone does not accelerate the synthesis of fat from protein though it does so from carbohydrate. When pyridoxin was added to a high-protein diet containing B_1 and B_2 and nicotinic acid, it was found that the loss of body fat could be prevented. These results indicate that pyridoxin may play an important part in gluconeogenesis.

In 1932, Blatherwick (191) and his collaborators noted the production of fatty livers when dried liver tissue was fed. The active factor was not identified but there was a tendency by Blatherwick and subsequent workers to attribute this result, in part at least, to the cholesterol contained in the liver (192).

Recently McHenry and his collaborators (193) have fed an alcoholic extract of beef livers. This material did not contain cholesterol but when added to a fat-free diet containing supplements of vitamins B_1, B_2, and B_6, it produced very fatty livers which contained large amounts of cholesterol. Choline had only a slight effect on this type of fatty liver but the administration of the pancreatic extract lipocaic, prevented the deposition. More recently these workers have shown that biotin produces the same type of fatty liver as does the liver extract (194) (195). Furthermore they have made fractions of liver, kidney, muscle, wheat germ, yeast, and rice polishings by the procedure used for the preparation of lipocaic and they found that all of these extracts were as effective as lipocaic in preventing the biotin type of fatty liver. They made the further interesting discovery that the administration of inositol prevented the development of the biotin fatty liver in much the same manner as the extracts of pancreas or the various other tissues (196).

In a recent paper Engel (197) has reported that the addition of inositol to a purified diet containing an adequate amount of choline and in addition thiamin, riboflavin, pantothenic acid, pyridoxin, and corn oil, was necessary to reduce the liver fats to normal values. Further claims are also made for the necessity of pyridoxin even in the presence of choline to prevent the accumulation of fat in the liver in prolonged experiments.

These researches promise to shed a great deal of light on the intermediary metabolism of food materials and the effect of the members of the vitamin B complex on these interchanges. The comments of Griffith (198) and Mitchell (199) on this aspect of the subject are very valuable and serve to emphasize some limitations of this method of approach.

IX. The Relation of Choline and the Other Lipotropic Factors to the Kidney

Very important new evidence of the significance of the lipotropic factors in nutrition was obtained by Griffith and Wade in 1939 (200), when they noted hemorrhagic degeneration of the kidneys in young male rats fed an alipotropic diet. These investigators, using animals from 21 to 26 days of age, observed fatty livers within 48 hours. The amount of liver fat increases for the first 4 to 6 days, and during this time there is no interference with appetite or growth. The kidney changes appear between the 6th and 8th day and are accompanied by a rise in blood *N.P.N.* The rats appear sick and in some cases exhibit ocular hemorrhages and an extensive regression of the thymus.[3] Protein but not blood is found in the urine, and the excretion of phenol red and of inulin is greatly decreased.

It is of great interest that recovery from the kidney lesions takes place spontaneously in the animals which survive. Renal function improves rapidly and the repair is evident from gross examination of the kidneys. The signs of hemorrhage disappear but the recovered organs are enlarged and in some cases rough and scarred with a white incrustation. There is evidence (201) that the renal damage may persist for many weeks and that the glomeruli as well as the tubules may be involved. The liver remains fatty throughout the period of the recovery of the kidney (202) indicating that there is not sufficient choline made available to remove all signs of its deficiency.

By using arachin, which contains very little methionine, as the source of protein, Engel and Salmon (203) demonstrated hemorrhages in the adrenals, lungs, and myocardium as well as in the kidney and the eye. Under these conditions none of the animals survived. These findings illustrate dramatically the importance of a source of dietary choline in the maintenance of cellular nutrition. Weichselbaum (204) reported some years ago that fatal liver hemorrhages occurred in rats receiving neither cystine nor methionine.

Histologically, Christensen (205) and György and Goldblatt (206) have noted the following changes during the acute phase of the kidney lesions.

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[3] Christensen and Griffith, J. Nutrition (1942), have recently reported the results of a more complete study of the relation of choline to these changes in the thymus gland.

Vascular congestion and degeneration of the tubules are the principal lesions. Congestion of the peripheral cortical capillaries and the capsular blood vessels produces the enlargement and the deep red appearance. Hemorrhage is found only in the capsule and at the edge of the cortex. In the most severe lesions the glomerular and other blood vessels are congested. The renal tubules in the deep part of the cortex and in the outer part of the medulla are necrotic and are always filled with casts.

All of the kidney changes are prevented by the addition of 1–2 mg. of choline daily to the basal diet, while the liver lesions require more—4 to 6 mg. daily.

The addition of thiamin, riboflavin, pyridoxin, pantothenic acid, ascorbic acid, or vitamin K has no effect on the incidence or severity of the renal or hepatic lesions in these young rats (201). Nicotinic acid appears to oppose the action of choline, as does cholesterol—the latter the more vigorously.

Cystine aggravates the effects of an alipotropic diet on the kidney (207) but this is true only within a certain rather low range of cystine intake (208). Some evidence has been secured that this effect of cystine is a general metabolic one, compensatory for the low cystine intake, rather than a direct chemical or physiological antagonism between cystine and choline.

It is of great interest that triethylcholine, which prevents fatty livers but does not replace choline in promoting growth on an alipotropic diet containing homocystine, does inhibit the production of the kidney lesions.

Griffith and Mulford (209) made a careful study of the relative anti-hemorrhagic potencies of choline and betaine. Betaine is approximately one third as active as choline under their experimental conditions, and they have suggested that only one of the three methyl groups of betaine may be available. The methyl group of methionine is apparently effectively utilized. At present it is impossible to predict from a chemical formula what methyl group will be available from a compound which contains them. A general discussion of the relation of chemical structure to lipotropic, anti-hemorrhagic and growth-promoting activities has been given elsewhere.

Griffith (210) has reviewed the various possible explanations of this action of the lipotropic and anti-lipotropic substances on the kidney lesions and they need not be recapitulated here. Since all these materials cause kidney changes which apparently correspond extremely closely to those they induce in liver tissue it is reasonable to suppose that the kidney effect, like the liver change, is exerted through the mechanism of an altered rate of phospholipid "turnover." This may not involve transport of phospholipid from the kidney but may be concerned with the nutrition of the kidney cells.

X. THE LIPOTROPIC FACTORS AND CIRRHOSIS OF THE LIVER PRODUCED BY DIETARY MEANS

The favorable results obtained in 1937 by Patek (211) and in 1941 by Patek and Post (212) in the treatment of clinical hepatic cirrhosis by means of a highly nutritious diet supplemented by vitamin B concentrates, has undoubtedly stimulated further experimental work in this field. In 1939, György and Goldblatt (213) found that rats kept on a diet deficient in pyridoxin but containing thiamin and riboflavin, exhibited hepatic lesions. These continued to appear when pyridoxin was added to the diet but were prevented when yeast or Peters' eluate was provided. The hepatic changes which consisted of fatty degeneration, necrosis and sometimes hemorrhage, were not, however, consistently reduced under these experimental conditions. Basing his suggestion on the results of Neale and Winter (214) György suggested that the effective substance in yeast might be purine in nature.

Rich and Hamilton in 1940 (215) (216) were able to produce, by dietary means, a cirrhosis of the liver in rabbits which closely resembled the Laennec type in man. The characteristic change was scarring—the newly formed connective tissue either surrounding the lobules in bands or extending diffusely through them and breaking them up in an irregular manner. The dietary evidence indicated that these lesions were due to the lack of some substance in yeast but not specifically to the absence of B_1, B_2, B_6, or nicotinic acid. The fat content of the livers varied considerably and in general became proportionately less as the scarring process advanced. The occurrence of the lesions appeared to be independent of the fat or protein content of the diet. The fact that the diet was deficient in choline was appreciated by these authors. Machella and Maguire (217) using the Rich and Hamilton diet were not able to produce hepatic cirrhosis in rats.

In an extension of his work on fatty livers in depancreatized dogs, Chaikoff working with Connor produced cirrhosis in the livers of normal dogs by providing diets very rich in fat and administering alcohol in large doses (218). Fatty livers and cirrhosis were found in four of sixteen animals. Later (1940) similar lesions were produced by high fat diets alone (219). These authors believe that the accumulation of excessive amounts of fat persisting for long periods stimulates fibrosis to such an extent that a true cirrhosis, which may cause death of the animal, is produced.

We will not attempt to review here the literature in which the effect of excessive amounts of cystine in the diet on liver histology is described but a brief summary will furnish a groundwork for recent results obtained when both cystine and choline were provided. In 1927, Curtis and Newburgh (220) described very extensive interlobular hemorrhagic necrosis of the liver of rats after 4 days on an 8 per cent casein diet with 20 per cent of added cystine. Using a slightly lower protein intake, with 25 per cent fat and from 5 per cent to 10 per cent added cystine, Earle and Victor (221) in 1941 found that when the cystine feeding was prolonged a portal cirrhosis was produced. This apparently resulted from proliferation of connective tissue and of bile ducts, which is a part of the repair process after the hemorrhage and necrosis.

In a later paper (222), these authors noted that the liver lesions are proportional to the level of cystine in the diet rather than to the total amount of the amino acid ingested. They found that choline antidoted the effects of small amounts of cystine but not of larger ones. These results were confirmed by Webster (223) who produced lesions by diets low in protein and high in fat. Yeast in adequate amounts did not prevent these lesions but increasing the casein level, adding betaine or reducing the fat, did. The character and extent of the hepatic lesions thus differs considerably in these various investigations.

We now come to a more direct consideration of the effect of choline and other lipotropic factors on these hepatic changes. György and Goldblatt (224) found that 10 to 20 mg. of choline daily appreciably reduced the incidence and severity of liver damage on a 10 per cent casein, 22 per cent fat, diet. The effect was much more dramatic when cystine was also fed. Choline and cystine together also protected the rat liver against the injurious effects caused by ingestion of dimethylaminoazobenzene (butter yellow) (225). By reducing the casein level from 18 per cent to 10 per cent and raising the fat intake to 22 per cent the liver injury could be produced regularly. These authors consider that the pathogenesis of dietary liver injury (necrosis and cirrhosis) is closely related to the lipotropic effect of casein and to fatty infiltration of the liver.

Blumberg (226) noted cirrhosis in rats on a high fat, low protein diet with added wheat germ and corn oil and later (227) on a diet lower in lipotropic factors (10 per cent casein and 55 per cent fat) he found that the cirrhosis developed to a marked degree within 125 to 150 days. *dl*-methionine (25 mg. daily) inhibited the development, and 1 per cent choline chloride prevented it.

It is important to note that choline alone as well as in combination with methionine or cystine prevented the development of cirrhosis in Blumberg's experiments.

Daft, Sebrell, and Lillie (228) produced cirrhosis in less than three months in rats on a low protein, low fat diet with only 0.5 per cent added cystine. Choline, methionine, and casein, either singly or in combination prevented this development. These substances exerted curative effects (229) but as would be expected the fibrous tissue persisted, while the liver cells regenerated and the gross appearance of the liver improved. The histological findings are described in a later paper (230). These workers also studied the effects of alcohol which aggravates the hepatic changes produced by dietary deficiency (231). Later (232) these authors presented evidence that on a 4 per cent casein diet choline alone prevented cirrhosis in the rats, cystine prevented the hemorrhagic necrosis while methionine inhibited the development of both conditions.

In 1942, György and Goldblatt (233) expanded and somewhat modified their previous theories on the relationship of the lipotropic factors to dietary cirrhosis. They stress the point that the factors predisposing to fatty livers—high fat, low protein diets, the absence of choline and other lipotropic substances and the presence of cystine—are the same as those which in more prolonged experiments lead to necrosis and cirrhosis. Furthermore, the preventive and curative measures are the same for both fatty livers and cirrhosis—casein, methionine, and choline in adequate amounts producing improvements in both cases.

These are indeed the main generalizations which can be made at this time, but a few other points may be mentioned. In all the work on lipotropic factors it has been apparent that more consistent results are obtained in rats than on rabbits or guinea-pigs. This is true in the study of dietary cirrhosis. Choline and the other lipotropic factors exhibit their effects best when the pathological condition is produced only by their absence from the diet, less dramatically or not at all when they are working against a toxic substance. Cystine may provide an exception to this rule since György and Goldblatt (233), Blumberg and McCollum (227) and Lillie, Daft, and Sebrell (231) all found that the combination of cystine and choline is more effective than choline alone. But here again in the presence of higher concentrations of cystine, choline is ineffective. The mechanism of this favorable effect of cystine with choline needs further study, but certain aspects of this point have already been discussed.

It is thus obvious that the work reviewed here establishes beyond doubt that the lipotropic factors are intimately connected with dietary cirrhosis. The relationship of this type of cirrhosis to that observed clinically is not as yet clear.

XI. Choline and Hyperplasia of the Forestomach in Rats

Pappenheimer and Larimore (234) in 1924 found hyperplasia of the squamous epithelium in the forestomach of rats on a white flour diet. The rats plucked and ate their hair, and hairs were found imbedded in the abnormal epithelium. Since no lesions were produced in normal animals after ingesting hair, the authors suspected a dietary deficiency. Many experiments have been devised to find this missing essential in the diet.

In 1937 (235) Sharpless produced the epithelial lesions by low protein and by white flour diets. Cystine was found to be protective against the effects of the former but not the latter, suggesting that the white flour diet caused the lesions not through its deficiency of protein but because of a non-specific factor (236). A combination of at least three members of the B complex is necessary to replace the deficiency in the white flour diet, namely, lactoflavin, nicotinic acid, and at least one factor other than B_1 in rice polish concentrate. Further work (237) on the known factors in rice polish concentrate revealed choline to be one of the essentials and pyridoxin, riboflavin, and cystine have recently been added (238). Choline or riboflavin supplements fed alone aggravate the lesions. Therefore as Sharpless points out, the actions of the protective factors are interdependent. He states also, that the epithelial injury seems to take place through the irritation of abnormally sensitive tissue by hair, harsh food particles, pepsin and hydrochloric acid or bile, and that the dietary supplements afford their protection by reducing the contact between the epithelium and these irritants and also by lowering its sensitivity to them.

Although the fundamental cause of the gastric epithelial changes still remains obscure, the action of choline in helping to maintain normal epithelium suggests a correlation between two observations previously made—namely, that protein tends to prevent (239) and fat to aggravate (240) the lesions.

XII. Choline, Butter Yellow, and Hepatic Tumors

When Sasaki and Yoshida in 1935 (241) and Kinosita in 1937 (242) reported that the feeding of *o*-aminoazotoluene or *p*-dimethylaminoazobenzene (butter yellow) would produce hepatic tumors in rats, a new field in cancer research unfolded. These investigations have brought to light the possibility of some extremely interesting, but probably indirect relationships of choline to carcinogenesis.

The basal diet used in these studies consisted of unpolished rice with a small amount of carrot. When butter yellow was added liver tumors were produced. The butter yellow must be fed for at least 32 days to produce cancer, and if it has been fed for 60 days, tumors will be produced even after it is withdrawn from the diet (249). The effects of various dietary factors have been studied.

Sugiura and Rhoads (243) (244) investigated the effect of yeast and of rice bran extract. They found that rice bran extract would prevent the hepatic tumors for a period of 150 days and 15 per cent brewers' yeast for as long as 284 days. If no protective measures were taken the lesions developed after 100 days on the diet and showed a greater incidence as the period on the diet increased. A supplement of 15 per cent yeast favorably influenced the accompanying cirrhosis, but when adenomatous hyperplasia of bile ducts, cholangioma, or hepatoma appeared, yeast exerted little influence. Kensler, *et al.* (245) reported that large amounts of riboflavin and casein inhibited the production of hepatoma on the rice-carrot diet. A supplement of liver (246) or replacement of rice by wheat (247) gave a marked reduction in the incidence of tumors.

György, Poling, and Goldblatt (248) replaced the basal diet of rice by one low in protein (6 per cent casein). Under these conditions they found that casein, and more particularly, the administration of choline and cystine afforded "definite but not regular protection against pathological changes in the liver."

Miller, *et al.* (249) replaced the rice diet by two types of ration: one designed to simulate the rice-carrot diet in terms of low protein and low vitamin levels, and the other low in protein (12 per cent casein) but adequate in other respects. With the latter type, the animals were maintained in better condition and the incidence of tumor production was satisfactory. These workers tested a great many different substances for effects on carcinogenesis by butter yellow. Casein (18–40 per cent), and yeast (31–51 per cent) offered partial protection; whole dried beef liver (20 per cent) and the water-soluble, alcohol-insoluble fraction of liver (1.2–2 per cent) provided nearly complete protection at 4 months (less at 6 months); and xanthine, *l*-cystine, *dl*-inositol and choline (0.1–0.5 per cent) gave no protection. After studying 34 diets, these authors conclude in general that: "the nutritionally adequate diets offered at least partial protection against hepatoma formation; (b) the protective supplements were usually rich in both protein and vitamin B complex, particularly riboflavin; (c) the non-protective diets were deficient in at least one of these factors."

White (250) found that butter yellow added to low protein diets retarded growth in young rats and the further addition of *l*-cystine or *dl*-methionine caused a prompt stimulation of growth. Low cystine diets (251) however, did not prevent tumor growth but did increase the latent period of their development. At 200 days 96 per cent of the rats on a high cystine-butter yellow diet had hepatic tumors, while at the end of 500 days only 60 per cent of those on low cystine diets had developed liver cancers.

Another link with the B-vitamins is found in the results of du Vigneaud's

(252) experiments with butter yellow. They seem to indicate that biotin may be carcinogenic when butter yellow is fed to rats in spite of a highly protective diet.

Jacobi and Baumann (253) connect choline with tumors through the phospholipid and through the labile methyl supply. Tumor tissue contains much phospholipid; phospholipid turnover is higher in tumors than in many normal tissues although not as high as in intestinal mucosa or liver. The lecithin of tumors has a higher rate of turnover than cephalin. Also, they suggest that butter yellow contains methyl groups which might enter into physiological transmethylations, and it resembles those substances which prevent kidney lesions in that its methyl groups are attached to nitrogen rather than to carbon. The demethylation of butter yellow destroys its carcinogenic power. These investigators found butter yellow to be effective in the prevention of the development of hemorrhagic kidneys (253).

It is obvious that only a beginning has been made in this interesting and promising field.

XIII. THE RELATION OF CHOLINE AND THE OTHER LIPOTROPIC FACTORS TO AVIAN NUTRITION

Under certain experimental conditions, a shortening and thickening of the bones, particularly noticeable in the tarsus and tibia of young birds, is produced when certain diets are provided. This condition is known as perosis. In many cases a distortion and dislocation of the hock joint results, and the slipping of the tendo calcaneus has provided the basis for the term "slipped tendon" disease. In 1936, it was shown that perosis may be caused by a deficiency of manganese. In 1940, however, Jukes (254) showed that when the supply of manganese was adequate, perosis was not prevented unless choline was also supplied in the diet.

The effect of choline in preventing perosis has been observed in both chicks and young turkeys (255) (256). The chickens need approximately 0.1 per cent and the turkeys about twice as much choline in the diet to protect them from this abnormal condition. It is most interesting that these birds are completely unable to utilize methionine as a substitute for choline (257).

There is a very interesting interrelationship between choline and glycine deficiency in so far as perosis is concerned. If glycine is added to the diet of chicks, perosis is produced; and if choline is then given, the condition is alleviated and growth greatly stimulated (256) (258). Jukes (256) has suggested as a possible explanation for these observations on the effects of glycine on perosis that the glycine, which is a precursor of creatine, prevents the appearance of muscular dystrophy. The perosis is not seen in the presence of the muscular dystrophy presumably because there is less pull of the muscles on the bones in this condition.

Jukes (258) has obtained evidence by adding certain analogues of choline to diets deficient in this substance, that the growth-promoting and anti-perotic properties of choline are truly distinct. He has found that methyl-diethyl-choline and beta-methyl-choline will protect against perosis but do not promote growth. On the other hand, betaine and betaine aldehyde promote growth but are devoid of anti-perotic activity for chicks. These results link choline with the metabolism of either muscle or bone—perhaps with both.

In rats, as we have seen, methionine and betaine are effective lipotropic substitutes for choline. It is of considerable interest that neither possesses anti-perotic activity. In the rat, both substances form choline in the body by methylation of aminoethyl alcohol. Apparently such a reaction does not occur in the chick. As Jukes points out, under certain conditions, choline is apparently a specific in the prevention of perosis.

Compounds other than choline derivatives under different conditions exhibit anti-perotic effects. Jukes (259) has shown that injection of a biotin concentrate into chicks on a raw egg white diet prevented completely the perosis which developed. Growth was improved, but the accompanying dermatitis was not entirely prevented.

XIV. THE PRELIMINARY WORK ON ACETYLCHOLINE PRODUCTION

The possibility that choline as a dietary factor, might affect the rate of formation of the neurohumor, acetylcholine, has been frequently discussed and some work preliminary to an extended study of this possibility has already been carried out (260) (261). In rats which had been for a long period on a diet deficient in lipotropic factors, the vagus nerve was carefully dissected out and stimulated under standard conditions. While the results in general were not satisfactorily consistent, it was found that in some series the vagus effect was less in the choline-deficient rats than in the controls receiving the same ration plus choline. Furthermore, it was possible to restore the vagus effect by the intravenous injection of choline in these animals. This problem demands a great deal of further work but it is hoped that more clearcut experiments can be devised as a result of the rapid progress which is being made in this field. By the use of gelatin or arachin, diets more nearly free of lipotropic factors can be planned. Guanidoacetic acid offers a further possibility of depleting the supply of lipotropic factors in the body. Experiments along this line are in progress in our laboratory. If a definite connection between the amount of choline or its precursors in the diet and the liberation of acetylcholine can be established this will become one of the most important aspects of this subject.

XV. The Influence of Choline on Lactation

Sure (262) has reported an influence of choline on lactation and normal growth of the young. When lactating rats were kept on a choline-deficient basal ration with various vitamin B supplements and cod liver oil the growth of the young was arrested at about the 13th day, signs of paralysis were noted and death occurred at about 17 days of age. 15 mg. of choline daily cured the paralysis and growth was resumed. If choline were given in the maternal diet alone, no beneficial effect was noted but a division of the 15 mg. supplement between mother and young caused the rate of growth to return to normal. Choline also was shown to be an essential nutrient for young rats after weaning.

XVI. The Effect of Choline on Blood Cholesterol and Experimental Atherosclerosis

Steiner (263), Baumann and Rusch (264), and Himsworth (265) demonstrated that choline has no effect on the hypercholesterolemia of cholesterol-fed rabbits. It has also been shown by Baumann and Rusch, and Himsworth, that choline has no effect on the cholesterol deposition in the liver or aorta of these animals. Steiner (263) concluded from a macroscopic examination that choline delays but does not prevent atherosclerosis in rabbits. Andrews and Broun (266) believe that choline exerts a protective action against atherosclerosis. Steiner has suggested that choline hastens reabsorption of the atheromatous plaques (267).

Huber, Broun, and Casey (268) reported that lipocaic is effective in preventing atherosclerosis in cholesterol-fed rabbits. The results of experiments by Andrews and Broun suggest that the potency of lipocaic may be due in part to its choline content but they believe that some other lipotropic substance may also be present.

It is apparent that some new approach to this aspect of the problem is urgently needed. The biotin and inositol studies may provide further leads.

XVII. The Question of Lipotropic Factors Other than Choline in Pancreas and Pancreatic Juice

During the insulin studies the lipotropic effect of raw pancreas had been noted. The findings by Best and Huntsman (269) that betaine was lipotropically active and that a similar effect was exerted by the protein casein (270) under certain conditions, indicated that several different types of compounds possessed lipotropic activity. Thus there was a possibility that the influence of the pancreas might be due to the presence in that organ of a protein or some substance other than choline with lipotropic activity. This possibility was not explored, however, and in 1936, Dragstedt, Van Prohaska, and Harms (271) reported that they had found a substance which permitted the survival and prevented or relieved the fatty changes in the livers of depancreatized dogs. These authors considered that the active substance, which they named "lipocaic," was a hormone. It has never appeared to us that their evidence justified this conclusion. Their work did show, however, that some factor in addition to choline was present in pancreas and that a part of the lipotropic effect must be attributed to this second factor or factors. Dragstedt (272) stated that it required much more choline than was present in an effective amount of pancreas to produce a comparable lipotropic effect. He felt that the pancreas exerted a specific effect which was not shared by other tissues. In this connection McHenry and Gavin (273) have shown that many other tissues may contain a non-choline lipotropic fraction which alleviates fatty changes in the liver of rats. Dragstedt and his collaborators did establish, however, that the pancreas exerted a lipotropic effect not attributable to choline.

Dragstedt's experiments have been criticized by Chaikoff and Kaplan on various grounds. It would appear, however, that the results of these two groups of workers are not in great disagreement. Entenman and Chaikoff (274) in a recent paper agree with Dragstedt and his collaborators that choline is not the only factor involved in the lipotropic activity of pancreas. They did find, however, that with 250 g. of raw pancreas a day they were able to prevent the deposition of fat in the livers of depancreatized dogs and the amount of choline contained in this pancreas was also effective. Choline is therefore an important member of the pancreatic lipotropic family. Kaplan and Chaikoff (275) (276) were interested in the blood lipid level as well as in the fat content of the liver of their depancreatized dogs which were maintained with insulin. They found that the ingestion of raw pancreas raised the level of blood lipids and lowered the liver fat but when the pancreas had been heated its effect on blood lipids was eliminated while that on the liver fat remained. These authors feel that the heat stable fraction is quite likely choline but they have not as yet identified the heat labile material. This heat labile fraction is secured by several extractions of the pancreas with acetone and ether. It should be borne in mind that substances which are heat stable in the pure state may be very labile if heat is applied in the presence of contaminating substances.

There has been considerable controversy over the effect of pancreas and pancreatic extracts on the so-called "fat" fatty livers of rats. Shapiro and Wertheimer (277) and Channon, Loach, and Tristram (278) have reported the presence in pancreas of a substance other than choline which decreased the fat content of the liver of rats. Aylward and Holt (279), MacKay and Barnes (280), and Best and Ridout (281) were not able to find any factor other than choline and protein in the pancreas which affected the fatty livers produced by feeding a diet low in choline and other lipotropic factors.

The experiments of McHenry and Gavin (282) who showed that choline was not effective in preventing the deposition of cholesterol and neutral fat in the livers of animals fed a liver extract or biotin are of great interest in this connection. They agreed that choline and protein were the only substances in pancreas which prevented the "fat" fatty livers. These findings may help to explain why some groups of workers failed to detect a lipotropic factor other than choline and protein but they do not offer an explanation why other groups, working with diets which presumably did not contain appreciable amounts of biotin, should conclude that a new substance was present. Subsequently McHenry and Gavin (283) showed that inositol exerted a pronounced lipotropic effect on the biotin fatty livers. Recently Owens, Allen, Stinger, and Dragstedt (284) have reported that although inositol does exert some lipotropic activity in depancreatized dogs, the effect is not as marked as the same amount of lipocaic. Inositol apparently exerts little effect on the signs of lipocaic deficiency other than fat accumulation in the liver. The situation is, therefore, that choline and protein (presumably methionine in large part) are the recognized factors in pancreas which exert a lipotropic effect in rats with the "fat" fatty livers. Inositol counteracts the biotin fatty livers but does not affect the "fat" fatty livers. The inositol content of pancreas and pancreatic extracts has not been determined. Dragstedt and his collaborators feel that there is still an unidentified component of pancreas, *i.e.*, in addition to choline, protein, and inositol which affects the deposition of liver fat.

Dragstedt and his collaborators concluded that lipocaic, the unidentified lipotropic factor in pancreas, was a second internal secretion. The evidence for this conclusion was that in their experiment dogs with pancreatic ligation did not develop fatty livers, and second that the administration of pancreatic juice to animals with fatty livers, produced no curative effect. In 1938, Ralli, Rubin, and Present (285) reported that the ligation of pancreatic ducts in dogs produced, in from thirteen to fifteen weeks, fatty liver changes which were essentially the same as those observed in depancreatized dogs. Montgomery, Entenman, and Chaikoff (286) reached similar conclusions. The same workers in 1940 (287) showed that the administration of 2 g. of choline daily completely prevented the appearance of fatty livers in the duct-tied dog. Later Chaikoff and his collaborators, Montgomery and Entenman (288) found that the daily administration of 400 cc. of pancreatic juice from normal dogs, prevented the deposition of liver fat in both depancreatized and duct-ligated animals.

It would appear, therefore, that it is not legitimate to assume, as Dragstedt and his collaborators have done, that ligation of the pancreatic ducts does not produce fatty infiltration of the liver. We have seen these fatty changes in duct-ligated dogs repeatedly in our own laboratory. Furthermore the evidence obtained by Chaikoff, Montgomery, and Entenman shows that pancreatic juice does contain a factor affecting deposition of liver fat. It may be remarked here also that pancreatic juice contains the heat labile fraction which Chaikoff, Montgomery, and Entenman found to be present in pancreas. This factor affects the level of blood lipids in depancreatized animals.

We may conclude that there is no evidence that the pancreas contains a second internal secretion and that the work of Dragstedt and his collaborators, and of Chaikoff and his group, shows that there is an unidentified substance in pancreas which affects fat metabolism.

XVIII. Choline Biosynthesis

In 1934, Best, Channon, and Ridout (289) studied the effect on liver "lecithin" produced by the addition of choline to three types of diet, first—a 60 per cent grain, 40 per cent fat ration; second—an 80 per cent grain, 20 per cent fat ration to which cholesterol was added; and third—a 100 per cent grain diet. The choline apparently caused a slight increase in the per cent of lecithin in the liver lipids of the first two groups and no significant change in the third. The "lecithin" was calculated from the phosphorus content of the ether-soluble fraction. In the first two cases the control livers were fatty and the livers of the choline-fed animals were not; in the third case, which was designed as a control, the livers of neither group were fatty. Choline did not influence the absolute amount of "lecithin" in the livers. In these experiments estimations of choline by biological assay showed that choline administration did not materially increase the proportions in which the choline-containing phospholipids occur in the phospholipid mixture.[4]

Beeston, Channon, and Wilkinson (290) interpreted the results of their experiments to mean that casein, but not choline, produced a slight increase in the absolute amount of ether-soluble phosphorus in liver lipids. These investigators did not determine the effect of casein on the choline content of the livers.

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[4] From the data given in the paper one may calculate the increments in total choline in the livers of the animals fed choline over those of the corresponding control groups. They were 55 mg., 61 mg., and 22 mg. respectively.

Jacobi, Baumann, and Meek (291) studied the total choline content of growing rats fed a basal choline-free ration which contained 18 per cent of egg white. Over an 8-week period as much as 76 mg. choline was synthesized by one animal. The amount of choline formed was somewhat reduced by a diet rich in fat but not influenced by (a) an increase in the amount of egg white, (b) the addition of cystine, (c) substitution of casein for egg white, or (d) the addition of choline to the diet. The increase in choline content was general throughout the body and was observed even in those cases where fatty livers developed. Since these rats received appreciable amounts of methionine, a now well-recognized choline precursor, the new choline was presumably formed by transfer of methyl groups to ethanolamine.

In a later paper, Jacobi and Baumann (292) reported the choline content of rats exhibiting signs of choline-deficiency to be slightly greater than in normal animals. Choline synthesis appeared to proceed during the period when the signs of choline-deficiency were exhibited. They concluded that the symptoms of choline-deficiency are due to a lack of a methyl-containing substance other than choline itself. There are, however, other possible explanations for their findings. The observation that young rats on a diet low in methionine lost weight but exhibited kidney hemorrhages which could be prevented by choline, indicates that with an inadequate supply of choline and its precursors, the active material is utilized in the prevention of kidney hemorrhages rather than in the promotion of growth. It would have been interesting to know whether a lipotropically equivalent amount of methionine would have promoted growth or exerted the same effect as the choline.

The use of tracer elements by du Vigneaud and Stetten has shown that choline is formed by the methylation of ethanolamine (293) (294). Methionine, betaine, and other lipotropic agents may act as methyl donors. Stetten (295) in a study of the rate of formation of choline, showed that the synthesis proceeds without hindrance even when the signs of choline deficiency are present.

In a more recent paper Stetten and Grail (296) have reported results on the synthesis of choline in rats on low choline diets which are in conflict with those of Jacobi and Baumann. Stetten and Grail found that the liver fat which results from dietary choline deficiency is poor in lecithin whereas that which results from feeding cystine or homocystine is abnormally rich in this phosphatide. Jacobi and Baumann determined choline by a colorimetric modification of the reineckate method. Stetten used a similar method for determining total choline but also did total lipid N and lipid P, from which he was able to calculate the amounts of lecithin and sphingomyelin. It is interesting to note that ethanolamine and serine produced no great increase in liver lipid but did cause a rise in the monoaminophosphatides (cephalin) of the liver.

In the hands of Stetten guanidoacetic acid has proved to be an anti-lipotropic agent with properties which make it a most useful tool in the study of the intermediary metabolism of choline (295) (296). By using it to deplete the labile methyl supply in the body of the rat, fatty livers were produced in which the choline-containing phospholipids, lecithin and sphingomyelin, were reduced to about one-thirtieth the normal values. However, it was also found that in the presence of even larger quantities of dietary guanidoacetic acid, the rate of choline synthesis (determined by methylation of isotopic ethanolamine) was not in the least impaired (295). Stetten and Grail (296) point out that the effect of ingested guanidoacetic acid is, therefore, to accelerate destruction of choline. It would appear that synthesis of choline, by methylation of ethanolamine, goes on at an undiminished rate, probably at the expense of available methionine. But as the labile methyl supply is drained away forming creatine (with a firmly bound methyl group), which is eventually excreted as creatinine, a condition is soon reached in which the newly formed choline has to give up its methyl groups to maintain the essential metabolic reactions. Thus in turn the choline reserves are depleted.

Although the precursors which are used for the biosynthesis of choline in the body of the rat have been identified, much remains to be learned about the process. An investigation of the mechanism of synthesis of choline in plant tissues might throw new light on the complex problem of choline formation and utilization in the body of higher animals.

XIX. The Mechanism of the Lipotropic Action of Choline and Other Substances

To describe the action of choline in the prevention and cure of fatty livers, it was suggested some years ago that the term "lipotropic" be used. This has now come into fairly general use and serves to distinguish the effect of choline on fat metabolism from its other physiological actions. We would now like to introduce the adjective "alipotropic" to describe diets free from choline, betaine, methionine, and other lipotropic factors. The term "anti-lipotropic" to describe the effect of cystine, cholesterol, and other substances may also be useful. It has already been used by Stetten.

In the very early experiments it was considered possible that choline acted through the formation of phospholipids (297). Phospholipid estimations, however, showed no increase in the amount of these substances in liver tissue. It was realized at the time that there might be a more rapid "turnover" and that this could happen without an increase in the amount present. The first proof that this actually takes place was obtained by Dr. Arnold Welch who had worked with us for a time in Toronto. Welch conducted a very ingenious experiment in which he fed arsenocholine and subsequently studied the arsenic content of the phospholipids. His arsenocholine possessed lipotropic properties similar to those of choline itself (298). The concentration of the arsenocholine in the phospholipids suggested that the dietary substance had entered into combination with the other components of the phospholipid molecule.

The recent paper by Welch and Landau (299) concerning the fate of ingested arsenocholine partially resolves some of the apparent discrepancies in the literature dealing with the several functions of choline and reopens the whole question of its mode of action in exerting lipotropic and anti-hemorrhagic effects. From the carcasses of rats which had been fed for one week on a diet containing 1 per cent arsenocholine chloride (without exhibiting signs of toxicity) they isolated the phospholipids. After hydrolysis with hydrochloric acid and removal of the fatty acids, the choline fraction was precipitated as reineckate, converted to aurichloride and recrystallized as such. Analyses for gold, nitrogen, and arsenic indicated that choline and arsenocholine were present in the ratio 1 to 14.2. The arsenic analogue forms derivatives similar to those of choline, but somewhat more soluble, so that some slight loss of arsenocholine undoubtedly occurred. The evidence seems clear cut, however, that the intact arsenocholine molecule is utilized in the biosynthesis of lecithin. The compound is completely inactive as a methyl donor. These facts coupled with its possession of marked lipotropic (300) (301) (302) and anti-hemorrhagic effects (303) suggest that arsenocholine is biologically active only as an intact molecule. It is, therefore, a logical assumption that choline also may exert its lipotropic and anti-hemorrhagic effects through reactions involving the intact molecule. There may be some support for the concept in the observations that the triethyl and diethyl-monomethyl homologues of choline, although inactive as methyl donors, possess lipotropic and anti-hemorrhagic properties.

Welch and Landau suggest that the available data may be interpreted in the following way: fatty livers develop when there is a deficiency of choline for synthesis of compounds concerned in lipid transport, and renal hemorrhages occur at a critical growth period when there is an acute deficiency of compounds required for the synthesis of material essential for cell structure. Not all of the metabolic reactions involved need be of an identical nature, and indeed there is already some evidence to support this.

Jukes (304), and Jukes and Welch (305) have found certain choline derivatives to be ineffective in preventing perosis in fowl, although possessing marked lipotropic and anti-hemorrhagic activity in rats.

Excellent evidence that choline acts by stimulating the formation of phospholipids was obtained by Chaikoff and his collaborators at Berkeley (306) (307). They used radio-active phosphorus as a tracer substance, and their analyses show quite convincingly that choline and the other lipotropic factors accelerated the rate of formation of phospholipids in the liver and other tissues, the site of the most active interchange being the liver. More recently Stetten (308) obtained similar results. He labelled the choline molecule with heavy nitrogen and, upon isolation of pure choline from the phospholipid of the bodies of the choline-fed animals, was able to detect a high concentration of the isotopic nitrogen in the choline.

As far as the synthesis of choline is concerned, the generally accepted precursor or "methyl acceptor" is ethanolamine, although it has not exhibited any lipotropic activity when tested (309) (310) (311). By feeding a series of compounds containing isotopic nitrogen (N^{15}) Stetten (312) was able to show that ethanolamine does indeed serve as a choline precursor. Its failure to exhibit lipotropic activity is not surprising since it could not form choline without a supply of labile methyl groups and the diets used in the tests were deliberately designed to be as free as possible of nutrients containing such units.

Stetten (313) fed labelled ethanolamine to two groups of rats, one on a diet sufficiently deficient in labile methyl groups to cause fatty livers, the other group adequately supplied with methyl. There was no marked difference in the amount of isotopic choline found in the phospholipids of the two groups. The result was the same on high protein diets as on low protein diets. Methylation of ethanolamine to produce choline proceeds unimpeded in the body of rats even when the supply of labile methyl is low. The choline content of the body is dependent to only a limited extent upon the amount in the diet, the level being maintained by biological methylation (314). However, metabolic processes dependent upon the presence of free choline are not favorably influenced by this biologically synthesized choline which is found combined in the tissue phospholipids. Stetten found that no ethanolamine arises in the body by demethylation of choline, and the nature of the demethylated product has not yet been discovered. The suggestion of Jacobi and Baumann (315) that choline must be oxidized before its methyl groups can be liberated fits many of the known facts. As the above authors point out, the physiological rôle of choline oxidase (316) has not yet been established and it may be significant that the two organs most obviously sensitive to choline deficiency, liver and kidney, contain the highest concentration of the oxidase. The observation that this enzyme is inhibited *in vitro*, by stearic acid (317) led Handler and Bernheim (318) to investigate the choline oxidase activity of fatty livers. A diet poor in methionine (arachin 15 per cent) and free of choline produced livers with a mean lipid content of 35 per cent in which the choline oxidase activity was markedly depressed.

Other activities of the liver are seriously affected when the animals are maintained for long periods on an alipotropic diet. Gluconeogenesis may be so inhibited that the depancreatized dog remains aglucosuric without insulin (319). In rats the ability of the liver to store glycogen and to eliminate injected dye (320) may be greatly reduced.

These results taken together, leave little doubt that the mechanism by which the lipotropic effect is produced, is by the stimulation of phospholipid interchange between the liver and other tissues. As mentioned above Stetten and Chaikoff found that the liver appeared to be the most active tissue in this interchange. Since in other studies on phospholipid metabolism, the conclusion drawn from the use of tracer substances has varied with the particular tracer used, it is of great significance in the investigation of lipotropic substances with three different labels, *i.e.*, arsenic, radio-active phosphorus, and isotopic nitrogen, the same answer has been obtained, namely, that there is an increased rate of phospholipid turnover.

The source of liver fat in the absence of dietary lipotropic factors varies under different conditions. It is well established that dietary fat may be deposited in the liver. Barrett, Best, and Ridout (321) could detect no transfer of previously deposited deuterio-fatty acids from depots to liver when diets low in lipotropic factors and consisting essentially of sugars were provided. Under these conditions when no fat was ingested, the liver fat was presumably formed from dietary carbohydrate. The studies of McHenry and Gavin, which have already been referred to, have led to similar conclusions.

The mechanism of the anti-hemorrhagic action of choline, betaine, and methionine is probably closely related to that of the lipotropic effect. Cystine and cholesterol which may counteract the lipotropic effect also interfere with the anti-hemorrhagic action. Choline accelerates the phospholipid turnover in the kidney (322) but to a lesser extent than in the liver. Since the anti-hemorrhagic effect is seen in tissues other than the kidney it is probable that this action is of a general nature and is concerned with the phospholipid metabolism of the cells rather than the mobilization of fatty acids, which is a very prominent rôle of the liver.

Choline promotes growth of young rats when added to a diet low in lipotropic factors (323). It also accelerates growth when added to an alipotropic diet containing homocystine, which by itself is neither lipotropic nor growth-promoting (324). In the latter case, as has been discussed elsewhere, the growth effect is due to the production of methionine from homocystine by utilization of the methyl groups of choline. However, since homocystine has not been isolated from food proteins or body tissues, it would appear that we must look elsewhere for the explanation of the growth effect of choline, which is produced when it is added to an alipotropic diet.

It makes relatively little difference whether choline is called simply a dietary factor, as we have termed it, a vitamin (György), or a vitagen (Rosenberg). We find much to support the positions which both György (325) and Rosenberg (326) have taken and it is obvious that some international group should, when such procedures are again feasible, make a ruling on the point. In the meantime choline should take its place with the other members of the B-complex from which it cannot now legitimately be separated in any complete consideration of metabolic changes.

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The Appraisal of Nutritional Status

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A. Introduction

Nutrition has taken its place as a fundamental science in medicine. Only in recent years has much progress been made in acquiring new knowledge of factors affecting the nutritional status favorably or adversely. In the early years of the 1900's, physicians attempted only to see that their patients received carbohydrates, proteins and fats in proper quantities and proportions. It was only later that the importance of minerals and vitamins was appreciated. Even then, attention and interest were only given to marked undernutrition and malnutrition. More recently, animal experiments have shown that the addition of the so-called protective foods to a diet already considered adequate increased the rate of growth and led to a significantly longer life. Dietary studies here and in Canada show that many people are not receiving food either quantitatively or qualitatively commensurate with the highest possible level of health. With the war, additional impetus has been given to the study of nutrition both in the individual and in population groups aiming to improve the physical condition of the fighting and the home fronts.

B. Prevalence of Malnutrition

The true prevalence of malnutrition is difficult to realize. It is true that relatively few deaths in the United States occur from pellagra or beriberi and that scurvy and rickets are seen less frequently than formerly; but, by newer standards, some degree of malnutrition has been found to be present in large percentages of all population groups studied.

I. EVIDENCE FROM MORTALITY STATISTICS

Relatively few people die of recognized nutritional diseases as the mortality statistics for the last available six year period show (Table I). This, however, is not wholly reliable since mislabeling, non-recognition of the nutritional factors causing death and the statistical practice of giving precedence to certain diseases over others, all play their parts in masking the true state of affairs. For instance, in the 2,569 deaths due to "alcoholism" in 1938, how many really died of beriberi and nicotinic acid deficiency, with alcohol only a contributory cause? How much beriberi is concealed in the 370,600 deaths in 1938 due to "diseases of the circulatory system," or in the 75,431 entered in the subclassification "diseases of the heart, unspecified"; how much deficiency disease is masked by the terms "senility," "cirrhosis of the liver," "psychoses," and possibly other headings? In the Psychiatric Division of Bellevue Hospital alone, for instance, at least 94 deaths that were recorded during a recent five-year period as due to "alcoholic encephalopathy" are now known to have been due to nicotinic acid deficiency (1). These deaths must have entered the official mortality statistics under the heading of either "alcoholism" or "psychoses." Even the apparent rising incidence of deaths from beriberi from 1 in 1933 to 42 in 1938 suggests that better recognition and diagnoses would give more reliable statistics and a clearer picture. Evidence based on hospital records are subject to the same criticism as that on mortality records (2). The recorded diagnoses, in addition to the factors mentioned, *i.e.* non-recognition, mislabeling and precedence given to certain diseases over others, are affected by two additional factors. The first is that the hospitalized population is not a representative group, because patients

TABLE I

Deaths from Various Causes in the United States Registration Area, 1933–1938

Cause of Death	1938	1937	1936	1935	1934	1933
Pellagra	3,205	3,258	3,740	3,543	3,602	3,955
Beriberi	42	21	11	7	5	1
Scurvy	30	27	33	30	36	28
Rickets	244	235	270	261	292	339
Alcoholism	2,569	3,305	3,714	3,349	3,655	3,297
Cirrhosis of the Liver						
Not Specified as Alcoholic	9,849	9,293	9,785	9,286	8,960	8,742
Specified as Alcoholic	959	1,037	802	797	773	607
Total	10,808	10,330	10,587	10,083	9,733	9,349
Dementia Praecox and Other Psychoses	1,369	1,576	1,678	1,383	1,468	1,449
Senility	9,242	9,904	11,367	10,010	10,961	11,318
Diseases of the Circulatory System	370,600	375,582	371,675	340,786	333,296	314,004
Unspecified Diseases of the Heart	75,431	68,959	66,307	55,192	49,986	44,321

with malnutrition enter the hospital only when sequelae develop and, on discharge, only the sequelae appear as the discharge diagnoses. The second is that certain anatomic lesions of malnutrition may be so prevalent that they are disregarded. This is paralleled by records of other highly prevalent conditions. According to Bellevue Hospital records in 1938, dental caries was recorded in only 0.68 per cent of its patients, whereas it is common knowledge that the incidence of dental caries in the adult population is near 90 per cent.

II. Evidence from Morbidity Rates

A more important aspect of this problem, however, is that nutritional diseases are rarely fatal, and that morbidity rates are high in proportion to the mortality. For example, Goldberger and his associates (3) estimated from a survey that there were at least 33 cases of pellagra for each death reported in 1917. Only pellagra with cutaneous manifestations was considered and because of this Sebrell (4) recently pointed out that this estimate would be too low for today. Improvements in treatment in recent years would modify further the morbidity-mortality ratio. Independently, Sydenstricker and Sebrell estimated that there were 100,000 cases of active pellagra in the United States in 1938 (5, 6).

Known and surmised data point to the importance of nutritional diseases and nutritional depressions. By nutritional depressions are meant the pre-anatomic states of pellagra, beriberi and scurvy, plus non-fatal anatomic lesions such as rickets, dental caries, cheilosis, xerosis conjunctivae and iron-deficiency anemias. Our problem, essentially, is not obvious deficiency diseases. As Tisdall points out, the man dying of tuberculosis is not the most important factor in the tuberculosis problem. The mild deficiency states are those which, over periods of years, affect resistance against disease, vigor and efficiency (7). Nutritional depressions are responsible for underweight, underheight, bow-legged, knock-kneed children; for much of the fatigability and chronic illness occurring in those ever-complaining adults in whom "nothing organic" can be found.

There are no exact statistics regarding the prevalence of "nutritional depressions"; but that they are present in significant samples of the population can readily be deduced from the medical, laboratory and dietary data included in nutritional surveys during the past few years.

An indirect medical survey of a portion of the adult male population being conducted by the National Selective Service shows that up to March 15, 1941, of one million men between the ages of 21 and 35 examined physically, approximately 400,000 have been found unfit for general military service; and in the opinion of Brigadier General Hershey (8) "possibly one-third of these are suffering from disabilities directly or indirectly connected with nutrition."

More direct medical surveys include, at the present time, as a rule, only the prevalence of dental caries, iron deficiency anemia, vitamin C undersaturation, and evidence of deficiency of riboflavin and vitamin A.

1. Dental Caries

Dental caries is one of the most widespread of human diseases. Borsook and Halverson (9) in a survey in Pasadena found in 80 individuals in 25 families in which 56 were less than 18 years of age that only 17 of those less than eighteen years of age had no dental caries. Harris (10) believes that 90 per cent of the civilized world is affected by this condition. Hollander and Dunning (11) found that in 12,753 employees of a large insurance company in New York, there were from 12 to 24 carious teeth in each person. At present, teeth account for nearly 20 per cent of the rejections by the National Selective Service. Klein and Palmer (12), in a study sponsored by the Milbank Memorial Fund found that in two groups of New York children examined, a private school group and a public school group, both were attacked about equally by dental caries, irrespective of the socio-economic difference between them. The only difference was that the well-to-do children received more dental care and therefore had less teeth extracted.

Although the cause of dental caries is unknown, most authorities agree that there is a definite relation to diet. The relationship between dental caries and each of many factors such as carbohydrates, vitamin C, vitamins D and A, the calcium-phosphorous balance, the alkali-acid balance have been studied. The only favorable effect seemed to come from a reduction of the carbohydrate intake and more especially of the sugars. Authorities agree, however, that it is when teeth are being formed, from before birth to 8 years of age, or 14 years of age if the three molars are included, that diet plays its very important rôle.

2. Underweight

Usually, in the middle and higher income groups, caloric requirements are met by the size of an individual's appetite. However, in different sections of the country, and in different socio-economic groups, this does not always hold true. In Youmans' (13) Tennessee study of 900 individuals, there was a high incidence of undernutrition. Hardy, Boyle, and Newcomb (14) in a study on approximately 7,000 children from widely different economic levels between the ages of 2 and 18, found that underweight occurred more frequently at low than at high income levels and more frequently in negroes than in white people.

3. *Iron Deficiency Anemia*

All surveys show a high incidence of iron-deficiency anemias, especially in the lower income groups. A study of 883 rural school children in Florida showed that 50 per cent were definitely anemic, while an additional 31 per cent were at border-line levels. This left only 19 per cent of the children with a satisfactory level of red blood cells and hemoglobin (96). Youmans (13) found anemia present almost entirely in women and children in the group he studied. Borsook and Halverson (9), on the other hand, in their California study found only one individual in 80 to have a hemoglobin value as low as 11 grams per 100 cc. This, however, concurred with the comparatively high level of iron consumption of this group revealed by the dietary survey done concurrently. In the Canadian studies (7) in Halifax, Quebec, Toronto and Edmonton, on about 75 families in each city, it was found that an iron-deficiency was present in many of the women, teen-age girls, and children under 11 years of age. The prevalence in children and women is explained by:

1. The fact that women eat much less meat than men.
2. The increased metabolic requirements due to growth in children and due to menstruation, lactation and pregnancy in women. Authorities all agree now that there is a greater incidence of low hemoglobin in pregnant women than in non-pregnant women of any average population group. Figures from 30 to 60 per cent have been quoted. However, work by Labate (15), by Becker, Bickerstoff, and Eastman (16), and by many others shows that hemoglobin levels are easily maintained at normal levels by the supplementary administration of iron. Labate, in 1939, showed that of 307 pregnant women with no iron therapy, 72 per cent had red blood counts lower than 4 million, with hemoglobin less than 11.6 grams per 100 cc.; while of 325 other pregnant women who had received iron therapy, only 28 per cent failed to meet these criteria.

4. *Vitamin A Deficiency*

Although there are extensive references, dating back to the early part of the nineteenth century, on the conjunctival and corneal changes which are now known to be associated with avitaminosis A, it was not until Kruse (17) showed that xerosis conjunctivae responded to oral administration of large amounts of vitamin A, that a reliable method for the detection of early signs of vitamin A deficiency was available. It is not meant to imply that the ocular changes are the only changes, for avitaminosis A is characterized by widespread epithelial changes throughout the body; but the eye is easily accessible for examination and gross and biomicroscopic study can detect all manner of changes, from the very mildest to the most marked. Kruse (17) suggests that night-blindness and adaptometer tests may not be indicative of changes as early as those shown by biomicroscopic examination. Recent work has shown that there is no direct correlation between dark adaptation tests and biomicroscopic examination. This may be explained in several ways:

1. As mentioned above, dark adaptation may be the result of a later change than the anatomic change seen biomicroscopically;
2. Changes in dark adaptation depend on more factors than just vitamin A;
3. Changes in dark adaptation may be due to a marked acute vitamin A deficiency, while the anatomic changes, which always take longer to form, seen by the biomicroscope may be the result of a chronic, though mild, vitamin A deficiency. While there is no definite proof yet of the above postulates, so-called "inconsistent" results by the two tests mentioned would fall neatly into place were they shown to be true.

Wiehl and Kruse (18) have shown that 86.6 per cent of 494 pupils in a public school group and all but one of 143 W.P.A. employees had some degree of xerosis conjunctivae. In 7.7 per cent of the public school group and in 45.5 per cent of the W.P.A. group, the xerosis conjunctivae was sufficiently advanced to form Bitot's spots, a condition considered to be a far advanced lesion due to avitaminosis A. On the wards of the Psychiatric Division of Bellevue Hospital, xerosis conjunctivae with grossly visible Bitot's spots occurs in about 60 per cent of all patients (19). The prevalence of these lesions is greater in colored than in white, in male than in female, and in old than in young patients. If the findings showing that any or all of these changes are due to a vitamin A deficiency are confirmed, the true prevalence of vitamin A deficiency will be many times greater than heretofore supposed. It may also be probable that the daily allowance of 5,000 units of vitamin A recommended by the Food and Nutrition Committee of the National Research Council may not be sufficient.

5. Riboflavin Deficiency

In 1940, Kruse, Sydenstricker, Sebrell, and Cleckley (20) showed evidence that the ocular changes of limbic vascular congestion and corneal vascularization were manifestations of riboflavin deficiency and that administration of large doses of riboflavin caused these manifestations to regress. Before this, medical surveys did not indicate the prevalence of riboflavin deficiency. Recently Wiehl and Kruse (18) reported that 2.3 per cent of a private school group, 75.8 per cent of a public school group, and 38.4 per cent of a W.P.A. group they studied, showed at least mild corneal vascularization. In a small group of 50 patients examined in the Mental Hygiene Clinic at Bellevue Hospital, all showed some degree of corneal vascularization. Riboflavin deficiency, on the basis of biomicroscopic examinations, is many times more prevalent than previously suspected, even if half the corneal changes were due to factors other than riboflavin.

6. Vitamin C Undersaturation

The prevalence of vitamin C undersaturation is high as is shown in reports on hospital and population groups. Vitamin C undersaturation, *i.e.* low plasma levels of vitamin C, while preceding low levels of vitamin C in the leukocytes, and periosteal and subcutaneous hemorrhages by a considerable period of time, indicates the necessity for dietary readjustment. A study of 157 consecutive patients admitted to the Medical Service of the Psychiatric Division of Bellevue Hospital in the fall of 1939 showed 42 per cent to have plasma ascorbic acid levels below 0.4 mg. per cent. Only 10 per cent showed levels of 0.8 mg. per cent or more. The cooperative survey in New York City reported by Wiehl and Kruse (18) found plasma ascorbic acid levels below 0.6 mg. per cent in 5.5 per cent of the private school group, 49.9 per cent of the public school group, and 55.8 per cent of the W.P.A. personnel. Borsook (9) found, in his Pasadena study, that none of his group had vitamin C undersaturation, and this is easily explained by the easy availability of citrus fruits and leafy vegetables. A dietary survey done simultaneously with the examinations showed that each individual obtained about 70 mg. of vitamin C daily.

7. The Prevalence of Rickets

With the development of knowledge of the cause and prevention of this disease, the prevalence of severe rickets has been greatly reduced in recent years. Twenty years ago, the prevalence of clinically detectable rickets in infants in some regions was estimated at about 75 per cent. Present figures indicate that the prevalence of active and healed clinical rickets is approximately 20 per cent of children of preschool age. This figure varies greatly from community to community, depending on urbanization, age, season of the year, race and the practice of preventive treatment.

III. Evidence from Dietary Surveys

Dietary surveys all concur in finding large numbers of unsatisfactory diets. The most extensive and comprehensive of these surveys, covering thousands of families of employed wage earners and clerical workers in cities, and non-relief, non-sharecropper families on farms, has been conducted by the Bureau of Home Economics of the United States Department of Agriculture and reported by Stiebeling and her associates in Circular No. 507 (21) and Miscellaneous Publication No. 405 (22) of the Department of Agriculture.

1. Expenditure for Food

From the reports of Stiebeling, *et al.* (21), most city families spent between 25 and 40 per cent of their income on food, and farm families spent between 34 and 64 per cent of their total family income for food (22). Analysis showed that nutritionally satisfactory diets were rarely obtained when the expenditure for food was below $2.50 per person per week. The weekly per capita food expenditure did not reach this level until the weekly per capita income exceeded $8.00 to $9.00. Data calculated from the report (23) on consumer incomes in the United States by the National Resources Committee indicate that 42.8 per cent of families of two, 65.9 per cent of families of three and four, 78.5 per cent of families of five and six, and 90.2 per cent of families of seven or more members had incomes, during the approximate period of these dietary surveys, permitting per capita expenditure for food of less than $2.50. These families, therefore, were probably not spending enough for food to provide a satisfactory diet.

Increased expenditures for food meant, of course, increased purchase of all food, especially the so-called protective foods, whose purchase increased in as great or greater proportion as the purchasing power. For example, families in North Atlantic cities spending $3.75 to $4.47 per person weekly for food purchased from two to three times as much butter, eggs, succulent vegetables, and meat, poultry and fish, as families spending from $1.25 to $1.87, while the figures for fruit were three to five times as high. This study by Stiebeling and Phipard (21) was made in 1935–37. Since then there has been a definite increase in the cost of living and estimates of costs need an upward revision if they are to be applied to 1942. On a smaller scale, Borsook and Halverson (9) studied the diets of 50 families in Pasadena with incomes from relief to those of $3,000, most of them between $1,200 to $1,800. The expenditures for food ranged from $.89 to $4.80 per person per week, with most of them less than $2.08. The diets of those people who spent less than $2.08 were inadequate as measured by present standards while the diets of those who spent more were nearly all adequate. The results agree fairly well with those of the large scale study of Stiebeling and Phipard.

2. Calories

Considering 3,000 calories as the satisfactory daily intake per requirement unit,[1] then if a corresponding intake is distributed in a family in accordance with the needs of each member, the diet would be adequate in terms of energy value. In the survey by Stiebeling and Phipard (22), an analysis was made between food expenditure and caloric intake. In the food expenditure range between $1.25 and $1.87 per person per week almost 40 per cent of the diets of city families furnished less than 2400 calories. With increasing expenditures for food, diets of higher energy value were reported. At the expenditure level $2.50 to $3.12 per person per week, only 12 per cent of the diets furnished less than 2100 calories a requirement unit a day, while at the $3.75 to $4.37 level, three-fourths of the diets provided more than 3600 calories a unit a day. Borsook (9) also found that more of the ample income families had diets of 3000 or more calories than those on restricted budgets. Twenty-two of the 50 families studied had less than 3000 calories per consumption unit per day, while 14 of these had less than 2700 calories per consumption unit per day.

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[1] The "requirement unit" is a standard "average" person—taken to be a moderately active man weighing 70 kg. The requirement (or consumption) of each nutritive element, whether calories, protein, or specific vitamins is thus expressed for each individual within a family or other consuming group on a scale related to the requirement of the standard unit, which allows for differences in age, body build and activity.

In a dietary study in Halifax in 1939, including records of 385 individuals in 82 families with family incomes between $450 and $1500 per year, Young (24) found the average consumption for men to be about 2600 calories (Canadian Standard—2800 calories), with, however, only 16 per cent under 70 per cent of the standard, and the average consumption for women about 2000 calories (Standard—2400 calories) with 11 per cent under 70 per cent of the standard. In another Canadian study in Toronto, Patterson and McHenry in 1939 (25) obtained records from 80 families comprising 313 individuals. The family incomes annually were between $1500 and $2400, a higher range than that of the Halifax survey. The eighty families, as a group, received on the average 93 per cent of the caloric standard (2800 calories). Twenty-eight per cent of the families were above their standard and only three families below 70 per cent of the standard.

Hunter and Pett (26), also in 1939, approached their survey in another Canadian city, Edmonton, in another way. Believing that a health hazard would in some cases be concealed by the family inventory method of food consumption, they obtained individual consumption records. In these families with incomes between $500 and $1500, the average daily consumption for the whole group was about 2000 calories, 87 per cent of the Canadian Dietary Standard. All the members in 11 of the 76 families studied received diets deficient in calories, while in the rest of the families, one or more members received diets deficient in calories. Of the whole group, only 47 per cent had diets adequate in calories.

3. *Protein*

From experimental evidence, it has been concluded that 45 g. of protein furnish the average minimum requirement for nitrogen equilibrium in a

70 kg. adult. A 50 per cent safety margin has generally been added to the average minimum in computing average requirements. The diet of 25 per cent of city families (Stiebeling and Phipard, 21) provided less than 70 g. of protein a day, with only 2 per cent of the diets furnishing less than 45 g. per day. At least 40 per cent of the protein usually came from "good" protein sources (milk, cheese, eggs and lean meats), and Stiebeling and Phipard concluded that both the quality and the quantity of protein in the diets were, on the whole, adequate.

Other studies (9, 24, 25, 26) concur in these results. Borsook found that none of his 50 families had less than 45g. of protein per unit per day, with only 12 of the families taking less than 66 g. per unit per day, and that more than one-half of the protein was of animal origin.

In the Canadian studies, none of the families in Halifax received in their diets less than the recommended average; in Edmonton, 12 per cent had diets grossly deficient in protein, most of these being mothers and children; and in Toronto in a higher income group, only 2 of 80 families were below the minimum of 45 g. per unit per day.

4. Carbohydrate and Fat

Carbohydrate and fat make up the remainder of the calorie-producing factors in the diet. The balance between the two are kept fairly normal in the surveys reviewed. Fat and carbohydrate as a rule were supplied adequately, especially where the total caloric intake was adequate.

5. Iron

Human iron requirements are not known exactly because of the difficulty associated with their determination. Sherman (27) considers 8 mg. daily sufficient to maintain equilibrium and recommends 50 per cent as a margin of safety, making 12 mg. the daily allowance. The daily allowance of iron recently recommended by the Food and Nutrition Board of the National Research Council is 12 mg. for men and women and 15 mg. for women during pregnancy and lactation.

In the survey by Stiebeling, *et al.* (22) many of the families had liberal supplies of iron in their diets. The diets of less than 5 per cent of the 4000 families studied failed to furnish as much as 10 mg. of iron per unit per day. However, in the low level of food expenditure (23), $1.38–$2.07 per unit per week, as many as 54 per cent of a group in some areas obtained less than 12 mg. of iron per unit per day. At the next higher expenditure level, $2.08–$2.76 per unit per week, only 15 to 25 per cent attained less than 12 mg. of iron per unit per day. This shows clearly the relationship of food expenditure level to the adequacy or inadequacy of this nutritive essential in the diet.

In the Canadian surveys (24, 25, 26), the daily iron intake was found to be grossly deficient, especially by the women and the teen-age girls. In Halifax, 44 per cent of the women, in Quebec, 32 per cent of the women, teen-age girls and children under eleven years of age, and in Edmonton, 90 per cent of the children under eighteen and 95 per cent of all the women in the study, received insufficient iron. Even in Toronto, where the next higher income-group, the middle income group, was surveyed, 44 per cent of the women obtained diets deficient in iron. The problem, evidently, is therefore one of both economy and education.

6. Calcium and Phosphorus

The average minimum requirements of calcium and phosphorus for maintaining equilibrium were concluded by Sherman (27) to be 0.45 g. of calcium and 0.88 g. of phosphorus for a 70 kg. man. The addition of a 50 per cent safety margin to allow for variations in the metabolism of different individuals and in the availability of the nutrients in different foods, brings the values of calcium to 0.68 g. per unit daily, and phosphorus to 1.32 g. per unit daily. Certain physiological conditions increase the requirements, for example growth in infancy, childhood and adolescence, and pregnancy and lactation in women. The recent recommended daily allowances by the Food and Nutrition Board of the National Research Council are given as 0.8 g. of calcium for both men and women, and for pregnant and lactating women 1.5 g. and 2.0 g., respectively. For children under twelve years of age 1.0 g. of calcium is recommended while 1.0 to 1.4 g. daily are recommended for adolescents and young adults between twelve and twenty years of age.

The average phosphorus intake in all the city groups (22) was above 0.88 g. per day per requirement unit, and below 1.32 g. only among families in the two lowest expenditure levels.

The data indicated that the diets were less adequate in calcium. About half the city families and 20 per cent of the farm families received less than 0.7 g. daily per requirement unit. The lowest four or five expenditure groups in all sections of the country were as a rule definitely deficient in calcium.

Borsook (9) found 5 of 50 families to have less than 0.45 g. of calcium per consumption unit daily and 16 families to have an intake between 0.45 g. and 0.66 g. daily. Twenty-one of the 50 families therefore had an inadequate intake.

Young in Halifax (24) found one-third of the women, 88 per cent of the boys and 91 per cent of the girls to be getting less than the minimum standard. This was substantiated by the average consumption of milk in the group, 1.5 quarts per capita per week.

The Quebec survey by Sylvestre and Nadeau (28), and the Edmonton study by Hunter and Pett (26) showed essentially the same thing, the marked deficiency in calcium intake by a very large percentage of the children, especially teen-age girls, and by the women.

The Toronto survey (25), on the other hand, showed a much better calcium intake by the family as a whole. However, the women and the girls between 11 and 18 years still took insufficient amounts. The somewhat higher income of this group and their greater consumption of milk explains this trend.

7. *Vitamin A*

The human requirement for vitamin A is unknown. The Food and Nutrition Board of the National Research Council has indicated that 5,000 international units for a moderately active man or woman would be satisfactory. This figure was probably arrived at by doubling the amount of actual vitamin A reported as necessary for good light adaptation (20 units per kg. of body weight) and then adding an additional safety factor to compensate for the fact that about two-thirds of the dietary intake of vitamin A is in the form of the provitamin, carotene, which is only 40 to 70 per cent as effective as vitamin A. Thus a dietary intake of the order of 5,000 units with two-thirds being derived from carotene represents not more than 3,000 units, probably less, of vitamin A esters. As indicated previously, light adaptation is probably a late manifestation of vitamin A deficiency, if it is one at all. The high prevalence of gross xerosis conjunctivae supports the belief that most American dietaries provide insufficient vitamin A to prevent this anatomic lesion. It thus seems to the authors that the daily vitamin A allowance should be considerably increased, possibly to as high as 7,500 or 10,000 international units of actual vitamin A per requirement unit.

All surveys show the prevalence of a serious deficiency of vitamin A in the diet. The Bureau of Home Economics statistics show that only 20 per cent of the city families obtain 6,000 or more international units of vitamin A per requirement unit daily, and only one-third receive 4,000 or more. Among the farm families, however, 60 per cent of the diets furnished 6,000 or more international units of vitamin A per requirement unit per day.

In the Halifax study, 34 per cent of the families, in the Quebec study, about 70 per cent of the individuals, and in the Edmonton survey, 67 per cent of the adults had diets containing less than 5,000 units of vitamin A.

8. *Vitamin B_1*

The vitamin B_1 requirement is known to be related to the caloric intake. Cowgill (29) and others have shown that diets containing a vitamin/calorie ratio[2] of less than 1.7 are almost always associated with a high incidence of clinical beriberi; that diets having a vitamin/calorie ratio of 2.3 or more are seldom associated with clinical beriberi; while diets varying between 1.7 and 2.3 are border-line in character, in that there are only occasional cases of beriberi (30). By converting Cowgill's vitamin/calorie ratio to international units and micrograms per hundred calories, we obtain the following figures:

Diets containing less than 8.5 international units or 25.5 micrograms of thiamin per hundred calories are associated with a high incidence of beriberi; diets containing 11.5 international units or 34.5 micrograms of thiamin per hundred calories are seldom associated with clinical beriberi; diets between these values are associated with only occasional cases of clinical beriberi.

If we apply these figures to the vitamin B_1 requirement on a 3000 calorie diet, we find that diets containing less than 255 international units of vitamin B_1 or 765 micrograms of thiamin may be considered definitely inadequate in thiamin.

From Stiebeling's data on thiamin intake, 5 to 22 per cent of city families at the food expenditure level \$1.25 to \$1.87 per unit per week received less than 255 international units (765 micrograms) of vitamin B_1. At higher expenditure levels, conditions are much better. The data groups families receiving from 240 to 399 international units; it is therefore impossible to give the percentage receiving less than 345 international units (1,035 micrograms) of thiamin. However, it can be said that those families receiving over 400 international units of thiamin per requirement unit are receiving, under normal physiological conditions, sufficient to prevent the development of classical beriberi. On this basis, at the expenditure level \$1.25–\$1.87, only from one-fifth to three-fifths of the families in different regional groups received an adequate diet as far as vitamin B_1 is concerned. At higher expenditure levels, however, the picture is much more satisfactory.

Borsook and Halverson (9) found only 2 families to have vitamin B_1 intakes of 340 international units, very little lower than the 350 unit minimum requirement. Fourteen additional families had less than 500 international units as their daily intake, while only 13 families took as much as or more than 750 international units of thiamin daily.

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[2] The vitamin/calorie ratio expresses the relationship between the caloric intake and the vitamin B_1 content of the diet. It is based on Cowgill's (29) requirement-prediction formula, and is obtained by dividing the vitamin B_1 milligram-equivalent content of the diet by the number of calories in the diet. Thus a diet of 3,000 calories containing 5,100 mg. equivalents of vitamin B_1 yields a vitamin/calorie ratio of 1.7. The milligram equivalent is equal to 0.05 International Units of vitamin B_1.

In Canada, surveys showed the deficiency of vitamin B_1 in diets to be much more serious. In Halifax, 100 per cent of the families consumed less than 350 international units per requirement unit, the per caput consumption being 210 international units. In Quebec, only the men had an average consumption of about 415 units of vitamin B_1, all the other individuals had an average intake below 350 units. In Edmonton, 70 per cent of the children and 77 per cent of the adults had grossly deficient diets (less than 300 international units) with regard to vitamin B_1, while only 7 per cent of the children and 5 per cent of the adults had adequate diets. In Toronto, 57 per cent of the families, at a slightly higher income level, had an intake of less than 350 international units. Individual intakes showed that 90 per cent of the people were considered deficient in vitamin B_1 (less than 500 international units).

Although the weekly food expenditures in the Canadian surveys are comparable to or even higher than the \$1.25–\$1.87 level in the Bureau of Home Economics surveys (considering the five year difference between the two sets of surveys and the rise in cost of living and the fact that the surveys were made in two different countries), the evidences of vitamin B_1 deficiency in the Canadian diets are even more striking than those in the American diets.

These figures reflect a really serious situation. Recent knowledge (29, 31, 32, 33) concerning thiamin shows that many symptoms are produced by deficiency and exist long before the development of clinical beriberi, a condition usually called polyneuritis or polyneuropathy in this country. These earlier symptoms are usually classified under the heading of a neurasthenic syndrome and consist essentially of the triad of anorexia, fatigability and sleep disturbances. When these symptoms exist in susceptible individuals for a period of time without correction other symptoms are produced, such as gas, palpitation, queer feelings in the abdomen, constipation, backache, headache, and all the other symptoms that are often associated with a neurasthenic syndrome. In order to prevent such a syndrome due to thiamin deficiency in all persons, a satisfactory 3000 calorie diet should furnish at least 600 international units (1,800 micrograms) of thiamin per nutrition unit.

The foregoing figures from the surveys assume a great significance in view of the standard of satisfactory thiamin intake recently set by the Food and Nutrition Board of the National Research Council which amounts to 600 international units per day per requirement unit.

9. Riboflavin

The daily human requirement for the other members of the B-factor are not well established. It is now considered that about 2.7 mg. of riboflavin constitutes a satisfactory daily intake of this vitamin. Here again, surveys by the Bureau of Home Economics and others in this country show that a marked dietary deficiency of this vitamin exists. Few of the city families in Stiebeling's study, at any but the highest expenditure level, received 2.7 mg. of riboflavin per requirement unit per day, while as many as 35 per cent of the farm families at the high expenditure level received less than 2.7 mg. of riboflavin. This supports clinical observations (34, 4, 32, 35) regarding the high prevalence of this deficiency in the population.

10. Niacin (Nicotinic Acid)

Quantitative data concerning the human requirement of nicotinic acid are crude and incomplete. The amount of nicotinic acid which constitutes a satisfactory intake is now thought to be about 18 mg. per requirement unit per day. The exact nicotinic acid content of the diets investigated in the Bureau of Home Economics survey could not be calculated at the time the reports were published. However, it was determined that the diets of city families in the South in the food expenditure level, \$0.63–\$1.12 per person per week, did not, on the average, contain enough nicotinic acid to prevent pellagra; while in the expenditure range, \$1.25–\$1.87 per person per week, the diets in Southern, Central, Eastern and Pacific cities contained only 10 to 40 per cent more nicotinic acid than the pellagra-preventive minimum.

11. Vitamin C

From metabolic studies, about 30 mg. of ascorbic acid were found to be necessary for equilibrium. About three times this amount is needed to keep the body saturated with it. However, no evidence is available which shows conclusively that saturation is necessary for a good state of health. Neither does a "satisfactory" blood level, since it is so dependent on the immediate recent intake, rule out scurvy. The Food and Nutrition Board of the National Research Council has recommended 75 mg. of ascorbic acid daily as a satisfactory intake.

In Stiebeling's reports, from 72 to 96 per cent of the diets in the expenditure group \$1.25–\$1.87 contained less than 75 mg. of ascorbic acid. At higher expenditure levels, although the number who received satisfactory amounts of vitamin C rose with the expenditure, there were still from 11 to 32 per cent of the diets at the \$3.75–\$4.37 expenditure level with ascorbic acid contents below 75 mg. per day.

Borsook (9) found every family in his Pasadena study to have 70 mg. or more of vitamin C daily. This is easily explained by the easy availability of leafy vegetables as well as citrus fruits.

Stiebeling found that although the diets of every family in the Pacific region in this study included at least 25 mg. of vitamin C per person daily, the percentages of those under 75 mg. a day equalled those in other regions.

The Canadian investigations (24, 25, 26) showed that the vitamin C intake was insufficient in their diets too. Between 60 and 70 per cent of their diets contained less than their standard, which is 60 mg. of vitamin C daily. The diets of the adults were found to contain less of the vitamin than those of the children.

12. Vitamin D

Little is known of the vitamin D requirement of adults and older children, although it is probably necessary. The Food and Nutrition Board of the National Research Council indicate the advisability of a daily intake of 400–800 international units for infants up to one year of age and for women during pregnancy and lactation. Amounts of vitamin D up to the minimal requirements for infants can be taken by adults where a good supply of sunshine is not available.

There are no dietary statistics available for vitamin D intake combined with its availability from sunshine. Its great importance in infancy and during pregnancy and lactation warrants the use of supplementary vitamin concentrates to insure a good quantitative intake.

13. Grades of Diets

The significance of many of these figures can be well appreciated when examining the summary of the survey made by the Bureau of Home Economics based on qualitative grading of the diets. Their specifications per nutrition unit per day were:

Protein	50 g.
Calcium	0.45 g.
Phosphorus	0.88 g.
Iron	10 mg.
Vitamin A	3,000 International Units
Thiamin	1.0 mg. (333 International Units)
Ascorbic acid	30 mg. (600 International Units)
Riboflavin	0.9 mg.

The diets of families were first classified (Table II) into three groups: poor, fair, and good. To be fair or good, the diet had to meet the above specifications; if it failed to meet the above specifications in one or more nutrients, the diet was classed as poor. To be good, it had to provide at least a 50 per cent margin beyond the specifications, and a 100 per cent margin in the vitamins.

The criteria used for a good diet are satisfactory, except in riboflavin, when compared with allowances recently suggested by the Food and Nutrition Board of the National Research Council (Table II).

Protein	75 g.
Calcium	0.68 g.
Phosphorus	1.32 g.
Iron	15 mg.
Vitamin A	6,000 International Units
Thiamin	2.0 mg. (666 International Units)
Ascorbic acid	60 mg.
Riboflavin	1.8 mg.

The criteria for a fair diet are significantly deficient in vitamin B_1, ascorbic acid and riboflavin, in that they are less than one-half the satisfactory allowances set up by the Food and Nutrition Board. The diets graded fair are therefore in many instances poor, and those graded good are in many instances capable of producing nutritional disorders.

TABLE II

*Distribution of Over 2,000 City and Farm Families at All Levels of Food Expenditure from All Regions in the United States, According to Grade of Diet**

	City	Farm	Total
Number of families	1,268	743	2,011
Per cent diets graded "good"†	7.9	51.0	23.8
Per cent diets graded "fair"‡	32.6	32.7	32.6
Per cent diets graded "poor"	59.5	16.3	43.6

* From data in Table 45, Stiebeling and Phipard (21), and Table 34, Stiebeling *et al.* (22).

† Diets to be graded "good" must contain protein 67 g., calcium 0.68 g., phosphorus 1.32 g., iron 15 mg., vitamin A 6,000 I.U., vitamin B_1 500 I.U., ascorbic acid 75 mg., riboflavin 1.8 mg.

‡ Diets to be graded "fair" (21a) must contain protein 45 g., calcium 0.45 g., phosphorus 0.88 g., iron 10 mg., vitamin A 3,000 I.U., vitamin B_1 250 I.U., ascorbic acid 37 mg., riboflavin 0.9 mg.

It should also be pointed out that the dietary calculations were based on food materials entering the home, without allowance for waste and cooking losses. Waste probably accounts for a 10 to 20 per cent loss, while cooking losses, particularly for ascorbic acid and thiamin, may be as high as 100 per cent.

It must be emphasized that the populations herein presented are families of employed (non-relief) wage earners and clerical workers in cities, and non-relief, non-share-cropper families on farms. Bearing this, and the populations to which they apply, in mind, it probably is not too high an estimate to say that at least half of our people are not receiving a diet that approaches satisfactory nutritional levels.

IV. EVIDENCE FROM FOOD SUPPLEMENTATION

Diets in Pregnancy

Studies of diets in pregnancy are important in revealing the prevalence of suboptimal nutrition in a population group whose health is of special concern to the community.

Ebbs, Tisdall and Scott (36) had exact records of the food intake during the first four months of pregnancy of women attending the prenatal clinic at the Toronto General Hospital. A group was selected which had been consuming a poor diet containing approximately 1600–1900 calories, 60 g. protein, 0.5 g. calcium and 10 mg. iron. This group was then divided into two parts. No change was made in the diet of the first. Those in the second were furnished each day with 1 egg, 30 ounces of milk, ½ ounce of wheat germ, 1 ounce of cheese, 4½ ounces of canned tomatoes, and 1 orange. The dietary improvement increased their calories to about 2600, protein to 100 g., calcium to 1.6 g., and iron to 24 mg. per day, besides materially raising the vitamin intake.

Williams and Fralin (37) conducted a similar study in Philadelphia. The study included 514 women chosen from the prenatal clinics of the Jewish Hospital, Presbyterian Hospital, and the Philadelphia General Hospital, from the Starr Centre Prenatal Clinic, and from the private practices of three Philadelphia obstetricians. The sample included white and negro women, private and ward patients, primigravidae and multigravidae from under 20 to over 36 years of age in different stages of gestation.

Eighty-four per cent of the women had caloric intakes below 2500 calories, 87 per cent received less than 85 g. protein, 97 per cent less than 1.5 g. calcium, 94 per cent less than 15 mg. iron, 96 per cent less than 9,000 International Units of vitamin A (more than half, in fact, had an intake of only 2,000–4,000 Units), 98 per cent less than 1.8 mg. thiamin (600 International Units), 91 per cent less than 70 mg. of vitamin C (over one-half received from 30–70 mg.), and 98 per cent less than 2.5 mg. riboflavin. From the few vitamin D values for food available, these women received from none to 140 international units daily. However, the frequent use of vitamin D concentrates in pregnant women insures, for that proportion in whom it is used, sufficient amounts of this factor.

Analysis showed that though, in many instances, the poor diets were due to economic circumstances, a lack of knowledge or poor diet habits was responsible for poor diets when the income was not low.

Although both studies, in addition to many others (38,39,40,41), concur in showing the very high prevalences of poor diets in pregnant women they do not agree on their relationship to complications antepartum, during labor, and postpartum (excluding, of course, those always associated with nutritional impairment, *e.g.*, neuritis, pellagra, anemia, etc.). However, the importance of improving the nutrition of this population group is agreed on by both. Improvement in nutrition of the pregnant women would bring about better maternal health, child health and development, and would also tend to improve the nutrition of the family as a whole (38).

C. Factors Contributing to Nutritional Failure

The circumstances mainly responsible for the nutritional failure in this country can be classified under four headings: poor food habits, highly milled grain and refined sugar, price rationing and intercurrent illness. Each of these factors is inextricably interwoven with the others.

I. POOR FOOD HABITS

Poor food habits develop partly out of economic factors, expressed as "price rationing,"and partly out of ignorance of the rules of good nutrition. Popular taste and social custom, however, account for the wide use of nutritionally inferior foods which include refined sugar, highly milled grain products, candy, alcohol and sweetened carbonated beverages. Poor food habits may also be negative. Non-consumption of adequate amounts of the protective foods is often due to failure to promote the taste for them in childhood, to local food customs, racial antipathies, and economic restrictions.

II. VITAMIN-FREE OR VITAMIN-POOR CALORIES

The rôle of highly milled grain products and refined sugar (white flour, white bread, highly milled cereals, granulated cane and beet sugar) has contributed enormously to the poor nutrition in our country. Food habits, appeal to popular taste, and economic necessity, since these foods are the cheapest source of calories, are closely bound up with their use.

Although transportation and refrigeration have had far-reaching effects by increasing the use of fresh fruits and vegetables in the American dietary in the last 100 years, the removal of 70 to 90 per cent of the B-factor and iron from flour and the increase of the yearly per capita consumption of sugar from about 10 to 100 lbs., has significantly reduced the amount of vitamin and mineral intake which is only slightly offset by the use of fruits and vegetables (Table III).

III. PRICE RATIONING

Stated simply, price rationing, the third factor involved in nutritional failure, means that about one-half of the people in this country have only three-fourths of the minimum cost of a nutritionally satisfactory diet available for expenditure on food. Like the other factors, price rationing does not stand alone, but is a part of and a cause of, poor food habits and the use of the highly milled grain products and refined sugar. This aspect of the nutrition problem properly belongs in the realm of economics, yet the physician and public health worker must take it into account in planning a nutritional program.

TABLE III

Vitamin and Mineral Content of Flour

	Whole Wheat*	White Flour*	Reduction	Enriched Flour†	
			Per Cent	Min.	Max.
Thiamin (mg. per lb.)	2.04	0.23	89	1.66	2.5
Riboflavin‡ (mg. per lb.)	1.13	0.18	85	1.2	1.8
Nicotinic acid (mg. per lb.)	12.3	3.7	70	6.0	9.0
Iron (mg. per lb.)	18.0	4.5	75	6.0	24.0
Calcium§ (mg. per lb.)	240.0	72.0	70	500.0	2,000.0
Phosphorus (mg. per lb.)	1,700.0	460.0	73	—	—
Vitamin D§ (I.U.)	—	—	—	250.0	1,000.0

* Council on Foods and Nutrition, American Medical Association (97).

† Committee on Food and Nutrition, National Research Council; and U. S. Food and Drug Administration.

‡ Inclusion in enriched flour and bread will not be required, but is optional, until July 1, 1942.

§ Inclusion in enriched flour and bread is optional.

IV. INTERCURRENT ILLNESS

The fourth factor responsible for nutritional failure is neglect in preventing and correcting malnutrition during and after illness. The part played by intercurrent illness and therapy in precipitating nutritional failure has scarcely been recognized. It is especially common in patients whose nutritional state has had little or no safety margin previously. In Table IV are listed some of the more important illnesses in which nutritional failure may occur.

D. The Recognition of Nutritional Inadequacy

With the growing recognition of the frequency of mild or early nutritional deficiencies, the old methods of using only anthropometric measure-

TABLE IV

*Conditions that May Contribute to Nutritive Failure**

- I. By interfering with food intake
    - 1. Gastro-intestinal disease
        - Acute gastro-enteritis
        - Cholecystitis and cholelithiasis
        - Peptic ulcer
        - Diarrheal diseases
        - Carcinoma of stomach and esophagus
    - 2. Food allergy
    - 3. Mental disorders, as
        - Neurasthenia
        - Neurosis
        - Psychoneurosis
        - Psychosis
    - 4. Operations and anesthesia
    - 5. Infectious diseases associated with anorexia
    - 6. Loss of teeth
    - 7. Heart failure (anorexia, nausea, and vomiting by visceral congestion)
    - 8. Pulmonary disease (anorexia, vomiting due to cough)
    - 9. Toxemia of pregnancy (nausea and vomiting)
    - 10. Visceral pain (as in renal colic, and angina that reflexly produces nausea and vomiting)
    - 11. Neurological disorders which interfere with self-feeding
    - 12. Migraine
- II. By interfering with absorption
    - 1. Diarrheal diseases, as
        - Ulcerative and mucous colitis
        - Intestinal parasites
        - Intestinal tuberculosis
        - Sprue
    - 2. Gastro-intestinal fistulae
    - 3. Diseases of liver and gallbladder
    - 4. Achlorhydria
    - 5. Carcinoma of the stomach
- III. By interfering with utilization
    - 1. Liver disease
    - 2. Diabetes mellitus
    - 3. Chronic alcoholism
- IV. By increasing requirement
    - 1. Abnormal activity, as associated with
        - Prolonged strenuous physical exertion
        - Delirium
        - Manic-depressive psychosis
    - 2. Fever
    - 3. Hyperthyroidism
    - 4. Pregnancy and lactation
- V. By increasing excretion
    - 1. Polyuria, as in
        - Diabetes mellitus
        - Diabetes insipidus
        - Long-continued excessive fluid intake, as in urinary tract infections
    - 2. Lactation

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* Taken from chapter on "Nutrition and the Deficiency Diseases," from Preventive Medicine in Modern Practice, Paul B. Hoeber, New York City, 1942.

ments and approximate estimates, by the presence or lack of subcutaneous fat and muscular firmness for diagnosis of nutritional status, became outmoded. More specific procedures for determining the adequacy of individual nutrients were needed. With the advent of many chemical and physiological tests, methods and procedures for detecting these early deficiencies were developed. They are dietary history, medical history, physical examination, and special examinations all combined.

I. DIETARY HISTORY

The dietary history should include diet habits of the individual and limitations of diet because of allergic conditions, food idiosyncracies, racial food preferences, or other likes, dislikes or excesses. In addition, a record of the total intake (the kind and amount of all food eaten) for at least three whole days should be examined. The average of the food values per day should then be compared to those recommended by the Food and Nutrition Board of the National Research Council (Table V).

II. MEDICAL HISTORY

The medical history, too, is important in evaluating a patient's nutritional status. Past or present illness may contribute to malnutrition in that decreased intake, impaired digestion, absorption or utilization, increased requirement or accelerated excretion, may deplete the nutritional stores which, in many cases, have been precariously near inadequate for a long period of time. In such a way, peptic ulcer, diarrheal diseases, sprue, disease of the liver or gall bladder, diabetes mellitus, hyperthyroidism, pregnancy and lactation are a few of the conditions which by history or physical examination can be etiologically indicated where malnutrition is being investigated. Therapy, too, as in the treatment of allergies, obesity, peptic ulcer and gall bladder disease, may contribute to malnutrition by nature of dietary limitations.

In the medical history, complaints such as easy fatigability, insomnia, anorexia, photophobia, nightblindness, burning of the eyes, lacrimation, burning of the tongue and mouth when tart foods are eaten, easy bleeding of the gums, palpitation of the heart, dyspnea, frequent eructation of gas, diarrhea, hyperesthesias, paresthesias or anesthesias of the fingers or toes, may be elicited. These must be evaluated and when all other causes have been ruled out, they may be suspected as nutritional in origin. In infants and children, the mother's story of the child's lack of appetite, lethargy, failure to gain weight and grow properly, irritability, or easy bruising may indicate a nutritional basis for these complaints.

TABLE V

*Recommended Daily Allowances for Specific Nutrients**

(Committee on Food and Nutrition, National Research Council)

	Calories	Protein	Calcium	Iron	Vitamin A†	Thiamin‡	Ascorbic Acid‡	Riboflavin	Nicotinic Acid	Vitamin D§
		g.	*g.*	*mg.*	*I.U.*	*mg.*	*mg.*	*mg.*	*mg.*	*I.U.*
Man (70 kg.)										
Moderately active....	3,000	70	0.8	12	5,000	1.8	75	2.7	18	
Very active..........	4,500					2.3		3.3	23	
Sedentary............	2,500					1.5		2.2	15	
Woman (50 kg.)										
Moderately active....	2,500	60	0.8	12	5,000	1.5	70	2.2	15	
Very active..........	3,000					1.8		2.7	18	
Sedentary............	2,100					1.2		1.8	12	
Pregnancy (latter half)..............	2,500	85	1.5	15	6,000	1.8	100	2.5	18	400–800
Lactation............	3,000	100	2.0	15	8,000	2.3	150	3.0	23	400–800
Children up to 12 yrs.										
Under 1 year ‖.......	100 per kg.	3–4 per kg.	1.0	6	1,500	0.4	30	0.6	4	400–800
1– 3 yrs.¶..........	1,200	40	1.0	7	2,000	0.6	35	0.9	6	
4– 6 yrs............	1,600	50	1.0	8	2,500	0.8	50	1.2	8	
7– 9 yrs............	2,000	60	1.0	10	3,500	1.0	60	1.5	10	
10–12 yrs............	2,500	70	1.2	12	4,500	1.2	75	1.8	12	
Children over 12 yrs.										
Girls, 13–15 yrs.......	2,800	80	1.3	15	5,000	1.4	80	2.0	14	
16–20 yrs.......	2,400	75	1.0	15	5,000	1.2	80	1.8	12	
Boys, 13–15 yrs.......	3,200	85	1.4	15	5,000	1.6	90	2.4	16	
16–20 yrs.......	3,800	100	1.4	15	6,000	2.0	100	3.0	20	

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* These are tentative allowances toward which to aim in planning practical dietaries. These allowances can be met by a good diet of natural foods; this will also provide other minerals and vitamins, the requirements for which are less well known.

† Requirements may be less than these amounts if provided as vitamin A, greater if chiefly as the provitamin carotene.

‡ One mg. thiamin equals 333 International Units; 1 mg. ascorbic acid equals 20 International Units (1 International Unit equals 1 U.S.P. unit).

§ Vitamin D is undoubtedly necessary for adults and other children; when not available from sunshine it should be provided probably up to the minimal amounts recommended for infants.

‖ Needs of infants increase from month to month. The amounts given are for approximately 6–18 months. The amounts of protein and calcium needed are less if from breast milk.

¶ Allowances are based on the middle age for each group, and for moderate activity.

III. Physical Examination

The physical examination covers the routine items with special regard to those usually associated with nutritional disorders.

Underweight, underheight and pallor, though not specific signs, are gauges of the general nutritional status. Edema may be associated nutritionally with a deficiency of thiamin or protein, or may be a manifestation of vascular, cardiac or renal disease. Special examinations, including urine analysis, serum proteins, thiamin concentration in the blood, blood pyruvate tests, x-ray of the heart, electrocardiogram, circulation time, and, in the routine examination, associated signs of varicose veins, thiamin deficiency or cardio-renal disease, help in differentiating the etiological factors.

1. *Vitamin A*

a) *Skin.* Follicular hyperkeratosis is now known to be a manifestation of vitamin A deficiency (42). The skin is dry and scaly and has a minutely horny appearance. The lesion is actually a keratotic plug in a hair follicle, which may have an unerupted hair buried under it, or a hair, broken and injured, protruding through it. In colored individuals, these plugs may be deeply pigmented, giving the skin an even deeper color than it is. Subsequently, with the plugs gone, the skin is left hairless with small crater-like follicles. When the lesion is more advanced, the skin between the follicles becomes keratinized. The areas most commonly involved are the anterior and lateral surfaces of the legs and thighs symmetrically and, somewhat less commonly, the same surfaces of the upper extremities. The back, abdomen and chest are very much less commonly involved.

b) *Eyes.* Xerosis conjunctivae (17), including Bitot's spots and the very much more advanced state, xerosis corneae, are manifestations of vitamin A deficiency. Although xerosis conjunctivae is not the only early change in avitaminosis A, the eye is a very convenient site for examining the changes which may be mirrored in other epithelial structures in the body.

Although microscopic examination by a biomicroscope and slit lamp is very valuable in that early changes may be noted and simultaneous examination of the corneal limbus for vascular changes due to riboflavin deficiency may be performed, gross examination is as valuable in detecting advanced lesions. Gross examination, moreover, is indispensable where the expense and unavailability of equipment are factors.

Normally, the sclerae are clear, bluish-white, the color of the choroid showing through the transparent, smooth conjunctivae. The vessels are small, unengorged, moderate in number. The caruncle and plica semilunaris in each eye are pale and flat. The very earliest changes may, many times, be seen only by the biomicroscope. Yet, if examined closely, the sclerae appear not quite so clear, a bit duller, a tinge of creaminess having supplanted the blue-white. Progressive changes are easier to see. The color of the sclerae change to yellowish-orange, taupe or gray-brown in some areas. The conjunctivae become irregularly heaped-up and loose, the uneven surface showing ridges and folds. The vascular network becomes more prominent, the vessels become engorged, more tortuous and larger in size. The plicae and caruncles become red, swollen and engorged with blood vessels. Progressively, the vascular network appears, paradoxically, to become less prominent, but as the conjunctivae become thickened, less translucent, more opaque, visibility of the vascular area becomes diminished. The conjunctival thickening becomes most marked near the canthus and the equator. Where the equator meets the limbus, as the condition continues, the conjunctival thickening becomes a Bitot's spot. The Bitot's spot is an elevated area, usually opaque, situated in its characteristic area on the equator near the limbus, usually triangular in shape with its base towards the limbus, although other forms have been noted, with various surface contours, flat, dome, ridged, etc., and colored white, creamy, yellow or orange, and is part of the whole process of conjunctival change, being one of the most advanced to occur.

Opinions vary as to whether all of these "spots" are due to vitamin A deficiency. Berliner (43) considers that many of the Bitot's spots may not be due to vitamin A deficiency and that many of the "spots" described by Kruse are really pingueculae, subepithelial infiltrations, with unchanged epithelium. On the one side are Berliner's contentions, with some of his work backed up by vitamin A plasma concentrations, on the other are Kruse's and others, with definite response to vitamin A therapy. That all of the "spots" are not due to vitamin A deficiency is probable in that all of them do not respond to specific therapy. However, time and additional clinical, histological and chemical investigative work will do much to settle points of dispute.

2. Vitamin B_1 (Thiamin)

The signs and symptoms attributed to vitamin B_1 deficiency are many and may be considered as (1) those symptoms similar to a neurasthenic syndrome, (2) those signs and symptoms involving the nervous system, (3) those involving the circulatory system.

a) *The Neurasthenic Syndrome.* Many etiologies have been suggested for the neurasthenic syndrome under which are listed complaints of easy fatigability, weakness, insomnia, anorexia, headaches, irritability, precordial pain, various aches and pains, poor memory and difficulty in concentration, and even additional complaints referable to the heart, bowels and genito-urinary apparatus. Many psychiatrists believe it is the result of physical factors. Psychotherapy has cleared up the complaints in some cases. Endocrines and vitamins, in turn, have been used in attempts to show their etiological relationship to this syndrome, but no one factor has been found which will satisfactorily improve the condition in every case.

Neurasthenic syndromes have been produced experimentally by diets poor in vitamin B_1 by Jolliffe and his co-workers (31) and by Williams and his co-workers (33). The complaints, moreover, were made to disappear in a short while by the addition of thiamin to the experimental diet. Similar syndromes, however, have been reported as cleared up by the administration of nicotinic acid (44), vitamin B_6 (45) and vitamin E (46). We should not assume, therefore, that the neurasthenic syndrome is due to thiamin alone, or to nutritional deficiency alone. However, there is definitely a group of individuals in whom the neurasthenic syndrome is a manifestation of thiamin or other nutritional deficiency and whose symptoms can be alleviated by specific therapy.

b) *The Nervous System.* Peripheral Neuropathy. The peripheral neuropathy of thiamin deficiency is a bilateral, symmetrical neuropathy involving predominantly the lower extremities. Unilateral neuritises are not, as a rule, due to a deficiency of thiamin, although it may, when it is involved, be an accessory factor. In its mildest form, the neuropathy is ushered in by paresthesias of the toes and perhaps also by calf muscle cramps, burning of the feet, and pains in the legs. Calf muscle tenderness and solar hyperesthesia are the earliest signs elicited. The hyperesthesia may extend up the ankles and legs in a sock distribution. These signs are only suggestive in the presence of a history of deficient thiamin intake, but when to these are added absent ankle jerks, a diagnosis of mild peripheral neuropathy can be made. Continued deficiency with spread of the lesion brings about loss of the knee jerks and posterior and lateral column signs consisting of impairment and then loss of vibratory sense and of position sense, and a positive Babinski toe reflex. Foot drop is the last development in this sequence of events. When in addition, the upper extremities or the brain is involved, the neuropathy is deemed severe.

Wernicke's Syndrome. The syndrome of ophthalmoplegia, clouding of consciousness, and ataxia was described by Wernicke in the 1880's, but it was not until 1938 that Alexander (47, 48) definitely showed the nutritional origin of this syndrome by producing it in pigeons on a thiamin-deficient diet. In 1940, he showed definitely that the disease occurring in man was identical with that in the pigeons.

Although Wernicke's syndrome has been seen most frequently in alcoholics, it has also been observed in others who have had concomitant illnesses which have interfered with their nutrition (gastro-intestinal disorders or carcinomas accompanied by cachexia and vomiting, pulmonary tuberculosis with associated vomiting, depressed patients who refused to eat, etc.) Wernicke's syndrome is a multiple deficiency disease. The ophthalmoplegia is due to thiamin deficiency and responds rapidly to specific therapy. The clouding of consciousness may be related to anything that interferes with proper brain metabolism. Among the known offenders are lack of carbohydrates, lack of oxygen, lack of thiamin, nicotinic acid and riboflavin, and probably lack of many other substances now under investigation. The stupor frequently responds to thiamin therapy but recovered cases often develop a Korsakov psychosis. The ataxia shows no such response and the factors implicated have not as yet been worked out.

Peripheral neuropathy has, in all but one of our series of 25 cases, accompanied the Wernicke's syndrome. We have attempted to explain it as an acute episode of complete deficiency of thiamin superimposed on a chronic mild deficiency. The episode of complete deficiency may be brought about by sudden increased requirement as by febrile disease, delirium, etc. This can even explain the occurrence of complete depletion occurring in the absence of chronic manifestations.

Korsakov's Syndrome. This psychosis consists of loss of memory for recent events, tendency to confabulate and disorientation for time, place and person. Although most frequently seen in chronic alcoholics, it has also occurred in other conditions (head injuries, arteriosclerosis, toxic and drug psychoses). Because peripheral neuropathy has so frequently been noted in patients with Korsakov's psychosis, thiamin has received attention as a therapeutic agent. Results conflict as to its effectiveness. We have not found it to be the answer. The rôle of thiamin has still to be determined.

c) *The Circulatory System.* The circulatory manifestations of vitamin B_1 deficiency may vary considerably. They are dyspnea on exertion, palpitation, tachycardia, often precordial pain and edema, and often dilated cervical veins, a palpable liver, and sometimes pulmonary congestion. They may occur in a person whose circulatory system is otherwise normal, or they may be superimposed on one previously damaged by degenerative, hypertensive or inflammatory disease. These manifestations may be classified as follows:

(1) Edema and serous effusions occurring in the absence of congestive heart failure, enlarged heart, or recognized etiologic factors producing edema and serous effusions.

(2) Edema and serous effusions occurring with supporting signs and symptoms of congestive heart failure, usually with definite roentgenographic evidence of cardiac enlargement.

(3) Sudden circulatory collapse which may be the first manifestation of circulatory failure or may occur after other signs of circulatory failure are well advanced.

The circulatory manifestations occur in about one-third of vitamin B_1 deficient subjects manifesting polyneuritis. They are more likely to occur in patients with suggestive or mild involvement than in those having advanced neuritis. This factor is related to the ability of persons with mild neuritis to perform muscular exertion.

Some of the more characteristic diagnostic features of the circulatory manifestations of vitamin B_1 deficiency are:

(1) Mild nature of the polyneuritis.

(2) Increased or normal velocity of the blood flow in the presence of congestive heart failure.

(3) Roentgenographic and electrocardiographic changes (although not diagnostic in themselves). These will be discussed later in the section on special examinations.

(4) Rapid response to specific therapy with complete and permanent reversibility of the circulatory manifestations.

3. Riboflavin Deficiency (Vitamin B_2)

a) *Skin.* In 1938, Sebrell and Butler (49) described the skin lesions due to riboflavin deficiency. These were the macerated fissures, usually bilateral, in the angles of the mouth, the lips abnormally red along the line of closure, a fine scaly, slightly greasy desquamation on a mildly erythematous base in the nasolabial folds, on the alae nasi, in the vestibule of the nose and on the ears. These were relieved by the administration of riboflavin but not of nicotinic acid.

The facial lesions we had noted in our patients (34) consisted of filiform excrescenses from the sebaceous glands, varying in length up to 1 mm., closely to sparsely scattered over the skin of the face. Their characteristic location was in the nasolabial folds, but in addition they occurred frequently on the alae nasi, occasionally on the bridge of the nose and sometimes on the forehead above the eyebrows. The skin on which the excrescences were located was the seat of a fine, scaly, greasy desquamation. On casual inspection, these filiform lesions resembled urea frost, but they could not be brushed off by rubbing with the fingers. The lips, particularly the lower, frequently showed a marked increase in the vertical fissuring, often without a break in the mucous membrane.

We maintained patients having these lesions characteristic of riboflavin deficiency on a diet poor in the B-complex and demonstrated that these lesions respond to synthetic riboflavin, but not to thiamin hydrochloride, nicotinic acid or vitamin B_6.

b) *Oral Manifestations.* A specific type of glossitis can be seen in some cases of ariboflavinosis. The tongue is smooth, the papillae flat or mushroom-shaped, the color purplish-red or magenta-hued rather than the scarlet-red of nicotinic acid deficiency. This glossitis may, not infrequently, be seen to develop in pellagrins, replacing the scarlet-red glossitis, under nicotinic acid therapy with insufficient riboflavin in their diets. Addition of riboflavin very quickly improves this glossitis.

c) *Eyes.* Kruse, Sydenstricker, Sebrell, and Cleckley (20) in a preliminary report early in 1940 described the ocular symptoms and signs associated with riboflavin deficiency in a small group of patients all of whom had cheilosis with or without glossitis at some time during the study and the beneficial effects of riboflavin therapy on them. Itching, burning and a sensation of roughness of the eyes with mild photophobia were common complaints and less frequently, severe photophobia, dimness of vision in poor light and partial blindness. Ocular symptoms had been described previously in patients with oral signs of ariboflavinosis and, although the symptoms were attributed to a deficiency of vitamin A, it was noted that the symptoms in a number of patients were relieved by riboflavin. Similar symptoms were also noted by Spies (1939) in 50 cases of malnutrition treated and relieved by oleum percomorpheum or carotene. Pock-Steen (1939) (50) described such symptoms and signs in 109 cases of sprue, in 78 of which they were relieved by the administration of riboflavin.

No report on the slit lamp examination of the cornea appeared, however, prior to the observations of Kruse, Sydenstricker, Sebrell, and Cleckley although the experimental background of Bessey and Wolbach (51) was suggestive.

The anatomic changes noted were circumcorneal injection, congestion of the bulbar conjunctivae, corneal vascularities and corneal opacities. Bessey and Wolbach (51) in 1939 had observed by slit lamp the corneal vascularization, infiltration and opacities in rats on progressive depletion of riboflavin, and occlusion and regression of the vessels and disappearance of some of the opacities soon after riboflavin therapy. Furthermore, they demonstrated these changes with india-ink injections of the vessels post-mortem. Johnson and Eckardt, following their observations of nutritional cataract in rats (52) and Bessey and Wolbach's demonstration of the relationship in rats between corneal vascularization and riboflavin deficiency treated rosacea keratitis with riboflavin and found dramatic results in 32 of their 36 patients (53).

Kruse, Sydenstricker, Sebrell, and Cleckley (20), meanwhile, observed that changes analogous to those in Bessey and Wolbach's rats occurred in human beings with riboflavin deficiency and that these changes were reversible with treatment. Their observations were enlarged (35) to 47 patients and careful examinations, including that done by slit lamp, were made. (Slit lamp changes will be discussed in the section Special examinations, p. 101.) In all cases, the functional disturbances, photophobia, dimness of vision, and ocular discomfort not improved after correction of refractive defects, were promptly, and sometimes dramatically, relieved by the administration of riboflavin. The corneal signs were likewise improved.

We know that injury to the eye, whether chemical, mechanical, due to light, etc., may cause vascularization and keratitis of the cornea. Whether other factors, in addition to these, can cause this vascularization is not known, but it is not entirely improbable. Riboflavin administration, over a period of 6 months, has not improved the corneal vascularization in all the patients studied in our group. It is entirely probable that this period of therapy is inadequate in many subjects whose lesions are chronic though mild. The relationship between the lack of riboflavin and other factors causing corneal vascularization is yet to be investigated.

4. Nicotinic Acid

Pellagra was first described by Casal, a Spanish physician, in 1735. However, it was not until Goldberger, in 1913, showed the relationship between diet and the disease, that the infection theory of its etiology was discarded. It was not until 1937 that the direct etiologic relationship of nicotinic acid and pellagra was shown.

The complete picture of partial chronic nicotinic acid deficiency, the scarlet-red stomatitis and glossitis, diarrhea, bilateral symmetrical dermatitis, and mental aberrations, forms a characteristic syndrome which is well known. It is not so well understood, however, that each entity may occur alone or in any possible combination.

a) *Skin.* The pellagrous skin lesions is well known as a bilateral symmetrical dermatitis which can involve any part of the body but usually involves first and most intensely those areas affected by trauma (in which category sunlight is also included). These areas are usually the dorsum of the hands, the wrists and elbows, the dorsum of the feet, the ankles, the knees, the neck, the face, the folds under the breasts, the inguinal and gluteal folds, the axillae, and the perineum, in the order of frequency of their occurrence.

The lesions start out with erythema and slight swelling. The swelling increases slightly and bullae form. After a period of time, the skin dries and desquamates either in large scales, or in small flakes, the erythema regresses and brown pigmentation ensues. The chronic lesions become thickened, indurated and deeply pigmented. The affected area is sharply demarcated from the rest of the skin. In the "wet" form, there is oozing of serous material from under the cracked scaling region.

These lesions respond fairly quickly to nicotinic acid therapy. Improvement is apparent within a few days and, depending upon the extent of the lesion, healing and regression occurs soon after.

b) *Mucous Membranes.* The changes in the mucous membranes are most easily seen in the mouth. The stomatitis and glossitis of nicotinic acid deficiency are very characteristic. In the fully developed picture the mucous membranes are scarlet-red, very often with superimposed gray patches of Vincent's infection. The tongue is often swollen, indentations made by the teeth being easily seen at the sides of the tongue. The papillae of the tongue become atrophic.

Nicotinic acid therapy dramatically changes the scarlet-red of the tongue to a normal pale pink quickly, but the regeneration of the papillae depends upon the length of time of their atrophy. In addition to this, the Vincent's patches clear up without local treatment when the underlying condition clears. It was not until the advent of nicotinic acid therapy for pellagra and this response of the glossitis and stomatitis to it were recognized, however, that these mucous membrane lesions without the dermal lesions were recognized as manifestations of pellagra; in other words, that "pellagra sine pellagra" was possible.

The mucous membranes elsewhere can undergo the same changes and these can often be visualized in the distal end of the gastro-intestinal tract and in the vagina.

Milder but more chronic signs appear on the tongue. These signs consist of redness of the tip and along the lateral borders, and after a time atrophy of the papillae in these areas. These signs may take several months to respond to therapy.

c) *Gastro-Intestinal Manifestations.* The symptoms included in the gastro-intestinal manifestations are vague and varying until very late in the disease. These symptoms may vary from burning of the tongue, pharynx, esophagus, abdominal discomfort, distention, even constipation, to nausea, vomiting and diarrhea, the last of which is a serious sign in the pathogenesis of the disease. Diarrhea in a pellagrin is serious and a sign for the institution of heroic measures. In addition to large doses of parenteral nicotinic acid, other members of B-complex are given orally and parenterally. We have found, in our debilitated pellagrins, many of whom were alcoholics, that diarrhea and dementia were the 2 D's very close to death.

d) *Nervous System.* Neurasthenic Complaints. Spies and his co-workers (54) state that pellagra patients are noted for their multiplicity of complaints, among which are many that are usually classified as neurasthenic. The most common of these symptoms are fatigue, insomnia, anorexia, vertigo, burning sensations in various parts of the body, numbness, palpitation, nervousness, a feeling of unrest and anxiety, headache, forgetfulness, apprehension and distractability. They noted that the "neurotic" symptoms showed a prompt response to nicotinic acid therapy. Probably of greater significance is their observation that these symptoms return when, without the patient's knowledge, nicotinic acid is withdrawn and another medicament of similar appearance is substituted for it.

Central Nervous System. The organic psychoses which complete the triad of diarrhea, dermatitis, and dementia are the more obvious and are better known. The most common, perhaps, is that in which loss of memory, disorientation, confusion and confabulation are present. There are also types in which excitement, depression, mania and delirium may occur. In our experience, a paranoid condition is common in pellagra as in many other organic psychiatric pictures. Spies and his associates (54) report that all their psychiatric patients recovered, but the psychosis in most of their cases was only of one to two weeks' duration. We can confirm these findings with our own experiences at Bellevue Hospital. We would, however, emphasize the fact that careful psychiatric examination reveals that these patients are frequently left with residual organic memory defects. In the psychoses of longer duration associated with pellagra, the response to nicotinic acid is certainly not spectacular, and specific therapy may not help at all. This does not mean that a lack of nicotinic acid was not important in the genesis of the mental picture. It does, however, point out the fact that these metabolic disturbances finally proceed to structural changes. When this latter stage is reached, the process may become irreversible. It must similarly be emphasized that many of the acute excitements and deliria associated with pellagra clear up without nicotinic acid therapy. Finally, pellagra patients are usually lacking in other factors contained in the well-balanced diet and probably necessary for normal brain metabolism. It is therefore suggested that adequate amounts of other vitamins be given to pellagra patients with encephalopathic manifestations in order to insure maximal therapeutic results.

Encephalopathy. Jolliffe, Bowman, Rosenblum, and Fein (1) have reported 150 cases of an "encephalopathic syndrome" heretofore almost invariably fatal, which they believe is caused by nicotinic acid deficiency. This syndrome may occur as the only manifestation of a deficiency disease or it may occur in association with pellagra, polyneuritis due to vitamin B_1 deficiency, or the ophthalmoplegia associated with Wernicke's disease. The clinical picture of this syndrome is more or less well defined and is characterized by clouding of consciousness, cogwheel rigidities of the extremities, and uncontrollable sucking and grasping reflexes. To be excluded are the encephalopathic manifestations of gröping, grasping and sucking which may occur in the course of delirium tremens, infectious diseases with delirium, expanding intracranial lesions, advanced cerebral arteriosclerosis and other diseases.

Since not all of the cases showing this syndrome presented the usual skin and mouth lesions associated with pellagra, it was assumed that this syndrome represents an acute complete nicotinic acid deficiency which develops so rapidly that the structural changes in the skin and mouth, characteristic of pellagra do not have time to occur. Patients manifesting this syndrome and treated by hydration or hydration plus thiamin hydrochloride almost invariably died (95 per cent); patients treated by hydration plus concentrates rich in the vitamin B complex showed a marked drop in mortality (50 per cent); but when these patients were treated by hydration plus nicotinic acid, the mortality fell to 15 per cent.

5. *Pyridoxin* (*Vitamin B_6*)

A syndrome specifically due to a deficiency of vitamin B_6 has not as yet been reported as a clinical entity. In rats, a deficiency of this vitamin is known to cause "rat acrodynia" foci of degeneration in striated and cardiac muscles, and changes in the nervous system, particularly of the columns of the spinal cord. Therapeutic effects have, however, been noted in several diseases of the nervous system, although its true position in the field of nerve tissue metabolism still remains to be determined.

a) *Paralysis Agitans.* Since muscular weakness and rigidity are characteristic of paralysis agitans, and since vitamin B_6 is involved in muscle metabolism, Jolliffe (55) tested its effect in this syndrome. He administered 50 to 100 mg. of pyridoxin intravenously either daily or every other day to 15 patients with paralysis agitans. Four showed subjective and definite objective improvement. Two were only subjectively improved. Of the 11 patients who showed no objective improvement, 10 had suffered disability for more than 3 years. Similar results were later reported by Spies, Hightower, and Hubbard (56). Jolliffe (57) subsequently reported a group of 32 ambulatory patients with Parkinson's disease. In the latter group, he noted improvement to occur in all groups of cases. These studies have been extended to 90 cases with permanent (6 months or longer) improvement in nine subjects. He concludes that the syndrome of paralysis agitans seems to include people who are helped by pyridoxin.

In a group of 12 chronic cases of paralysis agitans (including idiopathic, postencephalitic and arteriosclerotic) treated with large doses of thiamin, nicotinic acid, riboflavin, and pyridoxin, Loughlin, Myersburg, and Wortis (58) noted no objective changes for the better. Several of the patients claimed to feel better subjectively, but this was also true for several control patients who received injections of saline.

The reported material is still too meager too draw any definite conclusions, but the evidence at hand suggests that:

(1) The best results are obtained in cases of short duration.

(2) The number of cases helped is small (about 10 per cent).

(3) In the latter, there is no evidence of any lack of pyridoxin, and the general beneficial effects may be related to the general effect of pyridoxin on muscle metabolism.

b) *Pseudohypertrophic Muscular Dystrophy.* Antopol and Schotland (59) reported improvement in 6 cases of pseudohypertrophic muscular dystrophy treated with pyridoxin. The treatment was instituted because of the previous finding that foci of muscle atrophy developed in rats deficient in vitamin B_6. They believed that their results were due to enhanced muscle metabolism, and did not imply that this form of muscular dystrophy was due to a lack of vitamin B_6. On the other hand, Ferrebee and his co-workers report entirely negative results in 21 cases of muscular dystrophy (14 of the pseudohypertrophic type), and Wortis (60) has seen no essential change in 5 cases of pseudohypertrophic muscular dystrophy treated with adequate amounts of vitamin B_6. These contrasts in therapeutic results are difficult to explain and indicate that additional factors may play a part in the results obtained.

6. *Pantothenic Acid*

Pantothenic acid has been identified as a chick-dermatitis factor and an achromotrichia factor in rats. Graying of fur in black rats maintained on diets deficient in vitamin-B-complex was first described in 1938 by Morgan, Cook, and Davison (61). Addition of a "filtrate-factor," now more specifically known as pantothenic acid, restores the black pigmentation to the hair in rats. Recently Ansbacher (62) has pointed to an additional chromotrichia factor, para-aminobenzoic acid.

Woolley (63) has shown that on a purified basal diet, even in the presence of inositol, but in the absence of pantothenic acid, alopecia develops in mice, and that administration of pantothenic acid causes hair to grow back.

7. *Inositol*

Inositol has been shown by Woolley (64, 65) to be the anti-alopecia factor of mice. In addition to its being necessary for normal growth, inositol is one of two factors, the other being pantothenic acid, whose absence produces the "spectacled eye" in rats (66, 67). What relation these findings, with regard to pantothenic acid and inositol, have to human nutrition and deficiency we do not as yet know.

8. p-Aminobenzoic Acid

p-Aminobenzoic acid has been shown to be a chromotrichia factor for the rat and a growth-promoting factor for the chick (62). Here again, we may say that we do not know, as yet, whether this factor is necessary for human nutrition.

9. Biotin (Vitamin H, Co-enzyme R)

"Egg-white injury," an induced biotin deficiency, is caused by the binding of ingested biotin by a protein fraction of raw egg white, thereby preventing its absorption. It has been observed commonly and experimentally in animals when large amounts of egg white have been used in the diet. A severe generalized eczematous dermatitis involving the eyelids and lips is observed in these animals. Sydenstricker and his co-workers (68) observed the signs and symptoms of experimentally produced "egg-white injury" on 7 volunteers. Depression, lassitude, somnolence, muscle pains, hyperesthesia and localized parasthesia, marked anorexia and precordial distress, were similar to signs and symptoms of thiamin deficiency. A fine branny desquamatory dermatitis, a striking grayish pallor, and lingual changes characterized by atrophy of the papillae, either patchy or generalized, while the tongue remained pale, were observed during the "deficient period." Examination of the blood showed decreased hemoglobin concentration in spite of adequate iron intake. Parenteral treatment with biotin concentrate brought about prompt relief of the depression, muscle pain, precordial distress, anorexia and the ashen pallor. At the time of publication, insufficient time had elapsed for the observation of further changes.

10. Vitamin B Complex

There are still many factors of the vitamin B complex which are unknown. Vitamin deficiencies, we know, are usually multiple. Treatment of a deficiency syndrome with vitamin concentrates alone brings to light, at times, manifestations which do not respond to any one known vitamin alone, but do respond to the whole B complex.

11. Vitamin C (Ascorbic Acid)

Scurvy has long been known as a marked depletion of vitamin C in the tissues. It is manifested by a bleeding tendency; and hemorrhage into the skin and mucous membranes, hemorrhage into joints and muscles, and bleeding from the gums are very common.

If this condition has existed for a long while, the individual is anemic and his skin pale. The skin in a scorbutic may have many ecchymoses out of proportion to the trauma causing them. Hemarthrosis is manifested by a painful and sometimes enlarged joint. Healing of muscles extensively infiltrated with blood is sometimes attended with calcification and fibrosis leaving the muscle hard and rigid. This is known as "scurvy sclerosis."

The classical picture of scorbutic gums is well known. The genesis of this condition starts with slight swelling of the gums, slight redness at the gingival margins and oozing of blood upon slight pressure. Traumatic factors in the mouth, collections of tartar at the gum margins, other factors of constant irritation cause infection and subsequent necrosis of gingival points. Progressively, the gums recede from the teeth and the interdental spaces become larger. In a severe, acute case, the gums appear as markedly swollen, heaped up bags of blood, the gums around the lower incisor teeth being primarily and most severely involved. Within 24 to 48 hours, there is a remarkable response to vitamin C therapy and within a week the swollen gums may recede to normal size. It is very important to include dental therapy in treating scurvy.

Other general manifestations in scurvy may be epistaxis, pin-point hemorrhages in the gut with subsequent ulceration (peptic ulcer), melena, hematuria, menorrhagia or metorrhagia.

The relationship vitamin C bears to repair tissue is well recognized. It has been observed that even well-healed scar tissue has sometimes become disrupted in advanced scurvy. The rôle of vitamin C in speeding up the healing of wounds in a good number of individuals with vitamin C undersaturation has been noted, and delayed healing of wounds has been produced experimentally by its deficiency (69).

In the infant, skin and mucous membrane lesions are the same. Anemia is present in the chronic case. However, in the absence of teeth, scorbutic gums are not characteristic. The scorbutic infant is irritable, does not eat, sleep or gain weight properly. The limbs, most commonly those of the lower extremity, are intensely tender. The infant characteristically keeps his lower extremities motionless and drawn up. The slightest touch of these very tender extremities causes the child to scream. Later in the disease, subperiosteal hemorrhage and even epiphyseal separation occur and these can be seen by x-ray. Scurvy is not seen in breast-fed infants and has become very infrequent since the addition of orange juice to the diets of bottle-fed babies. However, although rare, we still have an occasional scorbutic infant admitted to the pediatric wards of our hospital.

12. Vitamin D

Vitamin D is one of the factors regulating the absorption of calcium from the intestinal tract. Vitamin D deficiency is manifested by rickets in the young, formative years, and by osteomalacia in adults. Although grossly, osteomalacia is not manifest, many of the rachitic changes are easily observable.

The bone manifestations by which rickets may be recognized vary with the age of the infant depending most probably on the variations in the growth and development of the bones and the varying activities of different age periods.

During the first eight-month-period of life, the changes in the skull are manifest. Because the infant lies on its head almost constantly, it develops soft-spots in the skull and this is known as cranio-tabes. Frontal and parietal bossing, developing usually at the end of the first year, gives the rachitic head a somewhat square appearance. Bending of the ribs at the costochondral junction (the "rachitic rosary") and costochondral enlargement at the ends of long bones (wrists, ankles) may appear as early as six months and may proceed in the ribs up to 18 months and in the long bones up to the fourth year. The thorax and spine may become deformed, due to sitting postures; the arms and legs bend due to weight bearing during active rickets. Pelvic deformities are common and rank among the commonest causes of dystasia. Fractures of the long bones are quite frequent in very severe cases of rickets and are due to osteoporosis of the bones.

Poorly developed, weak muscles which are present in almost all severe cases contribute to the fact that rachitic children seldom sit, stand or walk at the usual age. Pot-belly and constipation are two other symptoms due to loss of tone in the abdominal muscles and in the muscular walls of the stomach and intestine.

Infantile tetany may be a manifestation in a very severe case of rickets resulting from a disorder in calcium metabolism.

13. Vitamin E (α-Tocopherol)

Vitamin E is essential in nuclear activity in animals especially those involving cellular differentiation and proliferation (such as the developing embryo in the female rat). Its relation to function in human beings is not established. Therapeutically, however, it has been used in habitual and in threatened abortion with favorable results reported by some.

Experimental work (70, 71) has shown that in the experimental animal, deficiency of vitamin E results in paralysis. Further work (71, 72) pointed out that the nature of the changes might be either muscular or spinal cord changes. Utilizing these experimental findings Bicknell (72) and Wechsler (73, 74) each reported favorable results in amyotrophic lateral sclerosis treated with vitamin E. Wechsler suggests that the syndrome may have varying etiologies and that it is only in the group associated with a deficiency of vitamin E that favorable therapeutic results are to be expected.

Of greater significance is his reported observation that on two occasions when the administration of the vitamin E was stopped, the weakness returned, and when treatment was resumed recovery promptly resulted. While Bicknell (72) reported favorable results in 12 of 13 cases of muscular dystrophy, and Stone (75) reported excellent results in 5 cases with vitamin E and vitamin B complex, Wechsler (73, 74) failed to obtain any improvement. Finally, Sheldon (76), Wortis (77) and their co-workers have noted no objective improvement in a total of 27 cases of muscular dystrophy, 31 cases of amyotrophic lateral sclerosis, and 4 cases of progressive spinal atrophy treated with large doses of vitamin E, apparently for sufficient periods of time. These discrepancies would bear explanation.

14. Vitamin K

Vitamin K is one of the regulatory mechanisms in the clotting of blood. A deficiency in vitamin K is manifested by hemorrhagic tendencies. Vitamin K deficiency is associated with intrinsic liver disease, obstructive liver disease, and with the first few days of life in infancy. The administration of vitamin K to the infant, dramatically improves his condition. The administration of vitamin K in extra-hepatic biliary obstruction is also attended by improvement. In intrinsic liver disease, however, if any improvement does occur, it is only temporary.

15. Vitamin P (Citrin)

Vitamin P, a flavone, has been found to influence capillary fragility· Petechiae and hemorrhagic tendencies, similar to those seen in scurvy, are manifestations of this capillary fragility. These will respond to vitamin P administration while not responding to ascorbic acid.

16. Anti-Pernicious Anemia Factor

A combination of a macrocytic hyperchromic anemia, achlorhydria and nervous system involvement (from paresthesias, through combined sclerosis, to mental symptoms) presents the familiar picture of Addisonian anemia. Although the etiology is still unknown, the anti-pernicious anemia factor is absent in all cases. This has been found to be abundant in liver, and liver therapy, both parenterally and by mouth, has checked the disease and improved the symptoms and signs very dramatically (except for irreversible neurological changes). The relation between the causes of the disease and diet is still unknown, but diet has a very close association with therapy.

IV. SPECIAL EXAMINATIONS

Special examinations for the detection of malnutrition fall into two categories, one, those which can be done easily; two, those which require either special equipment, special training, or take long periods of time. In the first category can be included urine examinations, blood counts, x-rays, electrocardiograms, and simple and routine blood chemistries (serum calcium, phosphorus and phosphatase, plasma ascorbic acid, serum albumin and protein, blood prothrombin and blood chlorides). In the second, the more complicated examinations, blood vitamin A, blood vitamin B, blood pyruvic acids, and biomicroscopic examinations of the eye, gums and tongue for corneal vascularity, scorbutic changes and lingual papillary changes are included.

a) *Urine.* Urinary findings of albumin or glucose while pointing to diagnoses of constitutional disease suggest that secondarily the nutritional state is affected. The loss of albumin, if extensive enough, causes hypoproteinemia and anemia and eventually edema. In diabetes mellitus, carbohydrate and fat metabolism are affected. In polyuria, in diabetes mellitus and insipidus, and in genito-urinary infections, water soluble vitamins are excreted in the excess fluid.

b) *Blood Count.* A red blood count, hemoglobin (done by a reliable method) and hematocrit, wherever possible, are done. Hemoglobins done with a Sahli hemoglobinometer (14.5 g. = 100 per cent) are reliable. When large numbers are to be done, methods using a colorimeter are rapid and very accurate. Both sets of methods rely on the color change produced by the addition of acid to blood with the formation of acid hematin. Wintrobe's hematocrit method is a well accepted method and should be employed when convenience allows complete blood studies. With the above data, even mild anemias whether macrocytic hyperchromic or microcytic hypochromic can be detected.

c) *Serum Protein and Serum Albumin.* In general, methods for determining serum albumin and serum protein are not very satisfactory. However, we have been using the Levy and Palmer (78) method for our determinations. Nitrogen recoveries from serum with added ammonium sulfate run as close as 99 and 100 per cent. The method involves the digestion of the protein with formation of ammonium sulfate, the subsequent oxidation of this with equivalent amounts of hypobromite, substitution of the resulting bromide with iodide and iodometric titration. The method is probably too lengthy for large nutritional surveys, but where a laboratory is set up and time is not a factor, this method is excellent. The biuret method (85) is simpler, less time-consuming, and has been found to be satisfactory enough for this purpose. In large studies of apparently normal individuals, the incidence of abnormal findings of the serum protein and the serum albumin may run as low as one per cent. Even where diets are fairly bad, the incidence of this finding is very low. Apparently protein intake from any source must be very low and this deficient intake must continue for a fairly long while before the serum reflects this abnormal situation. In renal failure and in intrinsic liver disease, the protein and albumin are very frequently decreased.

d) *Blood Chloride.* This method consists simply of precipitation of the chlorides with a standard silver nitrate solution, the excess silver nitrate being titrated with thiocyanate solution. This method can be used in the few cases where edema is present and a differential diagnosis is to be made.

e) *Vitamin A.* Biomicroscopic Examination. The changes in the conjunctiva which can be seen grossly have been discussed. Biomicroscopic examination, of course, has the advantage that early deficiency changes are made easily visible, and that early improvement can also be noted. However, the unavailability of this instrument, in addition to its high cost, may not always make this procedure feasible. In any large study, however, the use of this instrument is a "must."

All the changes seen grossly are also seen, more exaggeratedly, with the biomicroscope. The differences in thickness of the conjunctivae result in three main degrees of transmission of light: transparency, translucency and opacity. A transparent conjunctiva appears as a clear medium through which the scleral landmarks and deep vessels can be seen distinctly. The vascular network is least extensive and least complex in pattern. A translucent conjunctiva appears as a turbid medium, the scleral landmarks and deep vessels being seen indistinctly as through a milky solution. The vascular network is more elaborate and extensive. There are gradations in translucence and visibility through the medium. With opacity, the deeper layers and vessels are not visible through the conjunctiva. The vascular network, although most extensive and elaborate, may appear, because of the low visibility, to be as inconsiderable as in transparent conjunctivae. Transmission of light is frequently not uniform for the entire conjunctiva of one eye nor even for the entire area in either the temporal or nasal zone. Transmission of light is often greater, too, in the superficial than in the deeper layers. A Bitot's spot shows more pronounced changes than in other areas of the conjunctiva, and in their early stages the biomicroscope can detect very early opaque spots even before they become elevated.

In addition, the biomicroscope makes visible the unevenness, the wrinkling, the roughness, the irregular thickening of the conjunctiva and the occasional conjunctival cysts.

Blood Vitamin A. Except for the spectrophotometric method, methods for blood vitamin A are not overly accurate since carotenes and carotinoids enter into the same reactions as vitamin A (80). Since the reaction of each with the reagent is different and their respective concentrations vary from individual to individual, their total effect is not always equal to the total concentration.

In spite of this difficulty, there is a definite place for the results obtained; that is, although not too much can be deduced from normal values, when low blood vitamin A values are obtained, a deficiency exists.

f) *Vitamin B_1*. Plasma Vitamin B_1. The methods used for vitamin B_1 concentration in the blood are yeast fermentation (81) and thiochrome methods (82). As an early gauge of deficiency the findings are not useful since these blood values have not been found to be decreased until gross evidence of thiamin deficiency is already present. Experimentally, however, it is a useful adjunct.

Blood Pyruvic Acid. Pyruvic acid is an intermediary production in carbohydrate metabolism. For the complete oxidation of pyruvic acid, vitamin B_1 is necessary. In line with this, it has been found that pyruvic acid is not well taken care of by the thiamin-deficient individual. This is the basis for the pyruvic acid test (83). Fasting blood pyruvic acids, in individuals with gross manifestations of thiamin deficiency, are elevated (84). In tests where large doses of glucose (about 100 g.) are given, and blood pyruvic acid values are determined at half hour intervals for four hours, normal curves rise to a peak at an hour and return to the fasting level within three hours. In grossly deficient individuals, the fasting level is higher. The curve rises to a much higher level, stays at this higher level, and does not return to the fasting level even in four hours (85).

It is too early to say yet, in the work we are doing on groups of neurasthenics, whether this can be used as an early gauge of vitamin B_1 deficiency, although some of our results show that it may be a possibility.

X-Ray Examination. The heart in the cardiac form o beriberi may be normal in size or much enlarged. The rapid reduction in the transverse diameter of the heart is rather dramatic with vitamin B_1 therapy. It is comparable to the effect of thyroid medication on the myxedema heart. Teleroentgenographic examination shows the enlargement of the heart to be chiefly of the right ventricle and auricle, and frequently, increase in the size of the pulmonary conus and pulmonary artery is noted. The left auricle and ventricle often contribute to the enlargement of the cardiac shadow, but as a rule, to a lesser extent than their mates on the right.

Electro-cardiographic Changes. Although few patients with the cardiac form of beriberi have normal electrocardiograms, the changes in the tracings are not usually characteristic. Shortening of the P-R interval (0.12 seconds or less) have been noted in beriberi in Java (86, 87). Others (88, 89) have noted various abnormalities, the chief among which are sinus tachycardia; flattening or inversion of the T waves, prolongation of the Q-T interval, low voltage of the QRS waves, depressed S-T segments, ventricular or auricular extrasystoles, and changes in the electrical axis either to the right or left.

Electrocardiographic changes have also been noted in subjects with experimentally produced thiamin deficiency (33). "They consisted of diminution in the amplitude of all complexes and particularly of the T waves of the chest leads. In certain instances the T waves became isoelectric or shallowly inverted. On restitution of thiamin to the diet the electrocardiograms became normal." These observations by Williams, Mason, Wilder, and Smith (33), confirmed the findings of Jolliffe, Goodhart, Gennis, and Cline (31), in whose experimental subjects precordial pain and electrocardiographic changes were likewise observed.

Circulation Time. In beriberi heart disease, an increased or normal velocity of the blood flow in the presence of congestive heart failure is a characteristic and diagnostic feature.

g) *Riboflavin.* Biomicroscopic Examination. As we have mentioned above, a biomicroscope is often not easily available. Some changes, however, when marked enough, can be seen either grossly, or frequently with a hand slit lamp. The corneal changes in riboflavin deficiency observed through the biomicroscope with the slit lamp have been well described and illustrated by Sydenstricker, Sebrell, Cleckley, and Kruse (35). The earliest signs by slit lamp inspection are described by them as marked congestion and proliferation of the limbic plexus. Arcus senilis and pigment at the sclero-corneal junction of colored individuals, when present, seem to mask the advance of the vessels into the corneal area. Progressing, very narrow capillary loops are formed which proceed through the narrow, formerly avascular zone of the sclera adjacent to the sclerocorneal junction. Very small capillaries arising from these loops send digitations into the cornea, and anastomose forming a tier of loops from which more capillaries arise. Both superficial and interstitial vascularization and subsequent opacities have been observed. In chronic, relapsing ariboflavinosis, Sydenstricker, Sebrell, Cleckley, and Kruse report observations of "universal vascularization and extensive superficial and interstitial nebulas."

h) *Niacin (Nicotinic Acid).* Biomicroscopic Examination. Kruse (90) has recently published his observations of changes in the tongue produced by acute and chronic niacin deficiency as seen through a biomicroscope. In the acute process, there is redness and swelling of the tongue, marginal indentations made by the pressure of the teeth, and finally smoothness. Vascular proliferation and hyperemia, hypertrophy and then their disappearance are the train of events occurring in the pathogenesis of the acute process in the lingual papillae. In the chronic form, the changes in the papillae go on from vascular proliferation and hyperemia, through infiltration to atrophy. The tongue itself shows crevices, fissures and loss of substance as the chronic process continues on its course. At any stage, an acute process may be superimposed on the chronic. From this, it can be seen that the pictures of niacin deficiency in the tongue may be multiple and varied.

i) *Vitamin C (Ascorbic Acid).* Plasma Vitamin C. The macrophotoelectric method (91) for determining the plasma vitamin C based on the reduction of 2,6-dichloro-phenolindophenol has been found to be both simple and reliable. Where an undersaturation of Vitamin C is to be determined, this method is sufficient. In the few cases where scurvy is suspected, the determination of the vitamin C concentration in the white blood cells, while somewhat more complicated, is more significant.

Rumpel-Leede Test. The Rumpel-Leede Test for capillary fragility is simple to perform but its value in diagnoses is not too great. In a positive test, pin-point petechial hemorrhages are produced in the skin distal to a blood pressure cuff which has been kept at the mean pressure between the systolic and diastolic pressures for five minutes. All that can be said for this test is that a large percentage of scorbutic patients show a positive test although a positive test does not diagnose scurvy.

X-Ray Examination. Roentgenographic changes in the diagnosis of scurvy in children are characteristic. Among the earliest changes are slight fraying at the distal ends of the radius, ulna, tibia, fibula, and the proximal end of the humerus. As the disease continues, there is increased density at the ends of the diaphysis; submetaphyseal rarefaction; thinning of the cortex of the diaphysis; lateral spurs at the end of the diaphysis; ground-glass appearances of the shaft, and epiphyseal separation. Subperiosteal hemorrhage usually occurs after most of these can be seen. It has been suggested that subperiosteal hemorrhage, which may be present, cannot be visualized until calcium salts are deposited in this area (92).

Biomicroscopic Examination. Changes in vitamin A deficiency, in riboflavin deficiency and in niacin deficiency as observed through the biomicroscope have been noted by Kruse. In addition to these, Kruse (93) has recently shown that early gingival changes associated with vitamin C deficiency can be detected with the biomicroscope.

In the acute process, the subsurface vascular papillae become engorged and dilated first, the gum itself then becomes red and next the reddened gum becomes edematous. Following this, there is recession of the gums with exposure of the enamel at the neck of the tooth, subsequent beginning formation of a pocket in this region often associated with secondary infection. These changes may be restricted to the interdental papillae, or may also involve the marginal gingivae or even the entire gum.

The changes seen in fully-developed scurvy have been described.

In the chronic process, the redness of the vascular reaction is soon obscured by edema and cellular infiltration so that the gum appears swollen and pale. Following this, atrophy becomes manifest by pitting, and then actual loss of substance. There is some retraction of the gum at the margin, gradual decrease in size of the interdental papillae going on to complete disappearance. In severe degrees of involvement, there is marked retraction of the gum at the neck of the tooth and excessive atrophy of the gum substance giving it a whitish appearance.

Here, too, an acute process may at any time be superimposed on a chronic state resulting in pictures of various combinations.

Vitamin D. Serum Calcium, Phosphate and Phosphatase. The significant and diagnostic chemical findings in rickets are low serum phosphate, high serum phosphatase, and usually a normal serum calcium. This increased serum phosphatase, with an initial lag, drops to normal with antirachitic therapy (94).

X-Ray Examination. With the aid of x-ray examination, rickets can be recognized before clinical signs have appeared and the activity of the process can be evaluated. The changes are noted at the lower ends of the radius and ulna because here they are most distinct.

In active rickets, the epiphyseal line becomes indistinct and irregular; the end of the shaft is fringed, and broadening and cupping occur early. As the disease continues, these signs become more prominent and the distance between the visible end of the shaft and the epiphyseal center appears greater than normal. Osteoporosis is evident.

With healing, the first sign is a linear shadow which appears a short distance beyond the end of the shaft on the epiphyseal side. As healing progresses, the area between these two becomes "filled in." The epiphyseal center of ossification becomes surrounded with a new area of calcification with a sharp border. Increase in density of the shaft also takes place but this process is very much slower. For determination of the degree of osteoporosis in the bones (perhaps as a measure of osteomalacia) *anterior-posterior* x-rays of the wrist and lateral views of the ankle can be made at constant distance and milliampere-seconds. A graded aluminum wedge is included in the film and the bone thickness is measured with a photoelectric cell densitometer against a constant source of illumination. The findings from this method, however, have not been found to provide much information, except occasionally in large surveys.

k) *Vitamin K (2-Methyl-3-phytyl-1,4-naphthoquinone).* Prothrombin Time. Quick's method involves the determination of the time required for clotting of recalcified plasma in the presence of an excess of added thromboplastin (95). Reference to a chart converts the result to prothrombin per cent of normal. Values above forty seconds, by this method, are definitely abnormal with a propensity for hemorrhage. Its usefulness in nutritional surveys is not great except, however, in groups such as expectant mothers. Its great importance also lies in determining the pre-operative status of individuals with liver disease.

E. Summary

The prevalence of malnutrition, from surveys conducted here and in several representative cities in Canada, is seen to be high.

More and more, emphasis is being placed on the importance of recognizing the pre-clinical states of malnutrition and to this end, newer methods are being sought and employed. Dietary and medical history, physical examination and laboratory techniques, and special examinations including biomicroscopic eye examinations, may all be employed, in so far as possible, in determining the nutritional status of one individual, or of a population group. The methods and the significance of their findings are discussed.

Careful examination and special techniques will do a large share in weeding out those without obvious manifestations of malnutrition but, nevertheless, with a subnormal status. Intensive specific treatment over long periods of time plus good diet, since deficiencies are more apt to be multiple; dietary education; eradication, where possible, of organic causes for "conditioned" malnutrition; and, in many cases, treatment of the socio-economic problems, would do much toward improving the happiness, physical well-being, and efficiency of large groups of people.

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Physical Methods for the Identification and Assay of Vitamins and Hormones

BY JOHN R. LOOFBOUROW

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I. INTRODUCTION

1. Methods to Be Discussed

Physical methods which have been employed in vitamin and hormone studies include: visible and ultraviolet absorption spectrophotometry, photochemical techniques, colorimetry, fluorescence spectroscopy, fluorophotometry, infra-red and Raman spectroscopy, x-ray diffraction studies, manometric techniques, polarography, and isotopic tracer methods. Of these techniques, some have had limited application and others are not universally regarded as physical. Since this discussion is limited to methods which are both essentially physical and of broad application in the vitamin and hormone field, only the following topics will be included: visible and ultraviolet absorption spectrophotometry, colorimetry, fluorescence spectroscopy, and fluorophotometry. It has not been practicable to consider all aspects of these techniques. In discussing colorimetry, for example, it has been necessary to omit reference to the many chemical problems concerned with the devising of color reactions and of procedures for eliminating interfering substances. In deciding what material to include and what to exclude, preference has been given to the quantitative and physical aspects of the methods discussed.

Of methods not considered, photochemical studies have played an essential rôle in the elucidation of the vitamin D problem (1), x-ray diffraction techniques have yielded useful information concerning the structure of vitamin C (2) and of sterols and related compounds (3), both manometric (4) and polarographic (5–10) methods have been used in assay procedures, and infra-red absorption techniques have been suggested for the assay of steroid hormones (11).

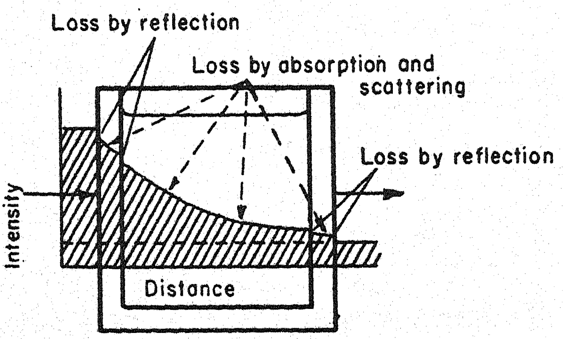

Fig. 1. Schematic diagram showing the losses of intensity in a light beam on its passage through a cell containing an absorbing solution

2. *Preliminary Considerations*

The techniques to be considered are based upon measurements of the optical properties of substances in solution. Fig. 1 illustrates a plane-parallel cell or cuvette filled with a solution. Suppose monochromatic radiation of wave length "λ" and intensity "I_0'" is perpendicularly incident upon one face of the cell. Let the optical path length between the inner walls of the cell be called "x", and the intensity of the transmitted radiation be "I_x'".[1] The incident radiation will be disposed of in the following ways: (1) by reflection at the various interfaces; (2) by scattering without change in wave length from particles in the solution; (3) by molecular scattering with change in wave length (the Raman effect); (4) by absorption and conversion to heat, chemical change, etc.; (5) by absorption and re-emission as fluorescent radiation "I_f"; and finally (6) by transmission through the cell and solution ("I_x'"). Permutations and combinations of these effects may occur.

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[1] In the absorption spectrophotometry of solutions, the symbol "x" customarily refers to the path length of radiation through the solution rather than to the total path length through solution and cell, and the symbols "I_0" and "I_x" refer to the intensity of radiation incident upon and emergent from the solution. Primes are used here to distinguish the intensities of radiation at the entrance and exit faces of the cell from the intensities at the entrance and exit boundaries of the solution.

Of the above effects, interfacial reflections and scattering are of interest herein only insofar as they may introduce errors into the measurements, and the Raman effect accounts for such a small fraction of the incident light that it may be neglected. Of primary concern, then, are the radiation "I_a" which is absorbed and degraded into other forms of energy or re-emitted as fluorescence, and the fluorescent light "I_f". The first of these can be determined only indirectly, by measurement of the ratio of "I_x'" to "I_0'" together with suitable correction for light lost by reflection and by absorption in the walls of the cell, etc.[2] The second, "I_f", can be measured directly.

The determination of I_x/I_0 as a measure of "I_a" at various wave lengths throughout a considerable region of the spectrum is known as "absorption spectrophotometry." If measurement is confined to a single narrow band of wave lengths, the procedure is "colorimetry" in the sense employed herein.[3] The qualitative examination of the fluorescent spectrum produced when a substance is excited by suitable radiation is called "fluorescence spectroscopy." The quantitative determination of total fluorescence, "I_f", without attempt at analysis of the fluorescence spectrum, is "fluorophotometry."[4] "Fluorescence spectrophotometry," the determination of the relative intensities of various narrow regions throughout the fluorescence spectrum, is a difficult and tedious technique and has had so little application to problems of interest herein that it is not considered.

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[2] Corrections for losses in intensity attributable to the cell, whereby I_x/I_0 may be obtained from measurements of I_x'/I_0', are discussed later.

[3] The term "colorimetry" lacks precision of meaning. It is applied by physicists to the designation of color sensations in terms of the stimuli which evoke them (12). It has been applied by chemists to the determination of the concentration of substances in solutions by the visual comparison of colors transmitted by solutions (*e.g.*, as in the Duboscq colorimeter). With the development of photoelectric "colorimeters," it has come to be applied to the determination of concentrations by measurements of the transmission of approximately monochromatic radiation at a wave length corresponding to the absorption maximum of the substance in solution. This last technique is essentially an "abbreviated absorption spectrophotometry." It has been suggested that the term "absorptiometry" or "absorptometry" be applied to it (*e.g.*, see Mellon (13)), but it continues to be called "colorimetry" by most workers.

[4] Also called "fluorimetry."

3. *Applicability of the Methods Discussed*

Absorption spectrophotometry and fluorescence spectroscopy have greatest use in the purification and identification of substances with highly-characteristic absorption or fluorescence. Their value in purification procedures arises from: (1) the saving of time in following the progress of purification by such methods rather than by biological tests, after a correlation between absorption or fluorescence and biologic activity has been established; and (2) the guidance as to the choice of purification procedures afforded by spectroscopic clues as to the probable chemical nature of the substance in question. Once the substance has been purified, spectra may be of material assistance in its identification and characterization. If the spectrum of the compound is sufficiently characteristic and the absorption or fluorescence sufficiently marked, the spectroscopic method may be used to determine the actual degree of purity of supposedly pure preparations. When it comes to the assay of crude preparations for small quantities of known vitamins, hormones, and enzymes, however, the spectrophotometric and fluorescence spectroscopic methods are usually inadequate either because they require too great quantities of material for practical assay purposes or because they involve tedious and time-consuming techniques.

Because of their lesser specificity, neither colorimetry nor fluorophotometry are as useful in problems of purification and identification as are absorption spectrophotometry and fluorescence spectroscopy. In assays of crude preparations, colorimetry and fluorophotometry have marked advantages arising from their greater simplicity and rapidity and in some instances from their materially greater sensitivity. Once the chemical nature of a vitamin or hormone has been established, the lack of specificity of these methods may often be offset by the development of procedures for eliminating the effects of the interfering substances most likely to be present in the crude preparations to be examined.

II. ABSORPTION SPECTROPHOTOMETRY

1. *Laws of Absorption and Methods of Presenting Data*

When monochromatic light traverses a homogeneous absorbing medium, the light transmitted by each succeeding layer of given thickness is a constant fraction of the light entering that layer. Thus if "I" is the intensity of light incident on the medium and the first cm. transmits 0.1 I, the second cm. will transmit 0.1 of the 0.1 I entering it, so that the intensity "I_x" traversing 2 cm. will be (0.1)(0.1 I), or 0.01I. The fundamental differential equation of absorption spectrophotometry is, therefore

$$-\frac{dI}{dl} = \alpha I \qquad (1)$$

in which the constant "α" is known as the "absorption coefficient." On integration between the limits $l = 0$ and $l = x$, this yields

$$\log_e \frac{I_x}{I_0} = -\alpha x, \text{ or } \log_e \frac{I_0}{I_x} = \alpha x \qquad (2)$$

an expression known as "Lambert's law." If this relationship is stated in $\log s_{10}$, thus

$$\log_{10} \frac{I_x}{I_0} = -Kx, \text{ or } \log_{10} \frac{I_0}{I_x} = Kx \qquad (3)$$

the constant "K" is known as the "extinction coefficient," after Bunsen and Roscoe (14), $1/K$ being the thickness which reduces the intensity of the transmitted light to $0.1I_0$.

For absorbing substances in solution, the absorption varies with the concentration as well as with the thickness of the solution traversed. In 1852, Beer (15) showed that absorption under such conditions is closely proportional to the number of molecules in the light path, whence

$$\alpha = \beta c \qquad (4)$$

where "β" is the absorption coefficient per unit concentration "c" Lambert's and Beer's laws may be combined in a single equation, thus:

$$\log_e \frac{I_0}{I_x} = \beta c x \qquad (5)$$

or

$$\log_{10} \frac{I_0}{I_x} = kcx \qquad (6)$$

Since confusion sometimes arises from the variety of ways in which the absorption of vitamins, hormones, and enzymes is presented, the various expressions and units used are summarized in Table I.

No exceptions to Lambert's law are known for strictly monochromatic light traversing homogeneous media. Deviations from Beer's law, on the other hand, are sufficiently frequent so that its validity should not be assumed until demonstrated experimentally for the substance in question within the concentration range of interest. Deviations from Beer's law are usually explained by the influence of concentration on the nature of the molecular species in solution (*e.g.*, the formation of polymers as concentration is increased, etc.). Other factors which can influence the molecules of the absorbing material, such as the pH and the nature of the solvent employed, may have profound effects on the spectrum, and the possible influence of such factors must not be neglected in quantitative absorption spectrophotometry. Finally, it is important to note that the Lambert-Beer law applies only to strictly monochromatic radiation. If this law is assumed to hold in colorimetric procedures in which poorly-defined bands of radiation are employed (for example, light limited by filters transmitting broad bands of the spectrum) serious errors may

TABLE I

Summary of Expressions Used in Absorption Spectrophotometry

Expression	Units Usually Employed	Nomenclature	Principal Application
$\beta = \frac{1}{cx} \log_e \frac{I_0}{I_x}$	x in cm. or mm. c in g./l. (mg./ml.), %, or g./ml.	β = Absorption coefficient.	Absorption of solutions—Continental usage.
$k = \frac{1}{cx} \log_{10} \frac{I_0}{I_x}$	x in cm. or mm. c in g./l. (mg./ml.), %, or g./ml.	k = Extinction coefficient.	Absorption of solutions—English and American usage.
$E^{1\%}_{1\text{cm.}} = \frac{1}{cx} \log_{10} \frac{I_0}{I_x}$	x in cm. c in %.	E = Extinction coefficient.	Absorption of solutions.
$\epsilon = \frac{1}{cx} \log_{10} \frac{I_0}{I_x}$ or $\epsilon = \frac{1}{cx} \log_e \frac{I_0}{I_x}$	x in cm. c in g. molecules per l. (moles).	ϵ = Molecular extinction coefficient.	Applicable only to known compounds. Useful in computing the combined absorption of substances present in known molecular ratios.
$\log_{10} \epsilon$, or $\log_e \epsilon$		Logs of molecular extinction coefficients.	Used in compressing data when comparing substances having widely different ϵ's on same graph.

Conversion Factors

$\epsilon = Mk$, where M = molecular weight, logs_{10}

$\epsilon = M\beta$, where M = molecular weight, logs_e

$k = 0.4343\beta$ $\beta = 2.3026k$

Note: The usual practice in absorption spectrophotometry is to express concentration in per cent as grams of solute per 100 ml. of solution.

result. If the incident radiation of intensity I_0 is made up of several different spectral bands of mean wave length λ_1, λ_2, λ_3, etc., of which the corresponding incident intensities are fractions A, B, C, etc. of the total incident intensity, and for which the extinction coefficients of the absorbing substance are k_1, k_2, k_3, etc., then

$$I_x = I_0(A10^{-k_1 cx} + B10^{-k_2 cx} + C10^{-k_3 cx} + \cdots) \qquad (7)$$

Under such circumstances, the transmitted light does not fall off in intensity as a simple exponential function of the distance traversed, or of the concentration of solution employed.

2. General Characteristics of the Absorption of Vitamins, Hormones, and Enzymes

The complexity of the band spectra of polyatomic molecules in general, and the diffuseness of the spectra of molecules in solution in particular, make the theoretical analysis of the absorption spectra of vitamins, hormones, and enzymes impracticable at the present time. The use of spectrophotometry to characterize such substances is, therefore, entirely empirical.

Two types of absorption are to be distinguished: "general" and "selective." Strictly, general absorption is entirely independent of wave length, but the term is usually applied to absorption which shows a progressive change as a function of wave length without exhibiting maxima or minima. Selective absorption is characterized by complex changes in absorption as a function of wave length, involving one or more maxima and minima. Selective absorption is of use in identification and assay procedures, whereas general absorption is not. Only compounds containing unsaturated linkages exhibit appreciable selective absorption in the region of the visible and ultraviolet spectrum ordinarily investigated (8000 to 2000Å.). This restricts the applicability of absorption spectrophotometry to those vitamins, hormones, and enzymes containing unsaturated linkages. Tables II to V, which are summaries of the absorbing characteristics of known vitamins and of certain hormones, have been prepared in order to indicate those substances to which the spectrophotometric method is applicable and in order to bring together in concise form data useful as standards of reference. The data upon which these tables are based are discussed in later paragraphs.

The important influence on absorption of factors such as pH, nature of solvent, photochemical transformation, deviation from Beer's law, etc. has been indicated. The instances in which these effects are of particular importance are indicated in the tables.

TABLE II

Absorbing Characteristics of Water-Soluble Vitamins

Substance	Solvent and pH	Absorption Maxima and Minima			References and Remarks
		λ in A.	ε	$E^{1\%}_{1\,\text{cm.}}$	
Thiamin (B_1) mol. wt. 337 for B_1HCl	Neutral water or neutral EtOH	min. 2140 max. 2350 min. 2520 max. 2670 min. 3020	5,800 11,600 6,800 9,000 0	172 344 202 267 0	(28). Reference (39) gives max. 2350 A., 10,250; max. 2650 A., 6,000. Beer's law holds through wide conc. range (28).
	Water, pH 3.0	max. 2480	11,800	350	(37). Reference (39) gives max. 2430, 10,600, at pH 2.3.
Riboflavin (B_2) mol. wt. 376	Water	min. 2100 max. 2200 min. 2400 max. 2650 min. 3100 max. 3650 min. 4000 max. 4450 min. 5300	23,400 30,900 14,700 34,900 2,180 9,360 6,000 10,600 0	623 822 392 929 58 249 160 282 0	(41). Lumiflavin in $CHCl_3$ has a similar spectrum (40).
Pyridoxin (B_6) mol. wt. 169	Water, pH 2.1	min. 2550 max. 2910 min. 3140	200 6,800 0	11.9 402 0	(47). For absorption at intermediate pH's, see Fig. 5.
	Water, pH 6.8	max. 2550 min. 2830 max. 3260	2,800 1,100 5,500	166 65.1 326	
	Water, pH 10.2	max. 2450 min. 2700 max. 3115	5,600 1,200 5,850	333 71.0 346	
l-Ascorbic acid (C) mol. wt. 176	Water, dilute solutions	min. 2120 max. 2650 min. 3010	562 9,680 0	31.9 550 0	(52).
	Water, 20 mg./l.	max. 2650	8,800	500	(51). Unstable, especially in alkaline solution. Beer's law holds only for dilute solutions, up to ca. 25 mg./l. (49, 50, 51).
	Water, pH < 3, 20 mg./l.	max. 2450	9,680	550	
	Water, 10 g./l.	max. 2450	9,680	550	
	EtOH, 20 mg./l.	max. 2450	9,680	550	
Nicotinic acid amide mol. wt. 107	Water	min. 2400 max. 2600 min. 2900	1,910 4,460 0	178 417 0	(54).
Biotin	Water	Negligible absorption			(56).
Folic acid	Water	Negligible absorption			(57).

TABLE III

Absorbing Characteristics of Fat-Soluble Vitamins

Substance	Solvent	Absorption Maxima and Minima			References and Remarks
		λ in A.	ε	$E^{1\%}_{1cm.}$	
Vitamin A_1 (crystalline) mol. wt. 286	Ethanol	max. 3280	60,000	2,100	(58). $E^{1\%}_{1cm.}$ decreases to 1800 on standing.
		max. 3240	49,200	1,720	(59, 60). No appreciable change in spectrum due to standing or to irradiation with visible light according to this reference.
	Methanol	max. 3240	50,300	1,760	
	2-Propanol	max. 3240	48,300	1,690	
	Ether	max. 3260	50,500	1,770	
	Isooctane	max. 3260	47,500	1,660	
	Hexane	max. 3260	47,900	1,680	
	Cyclohexane	max. 3260	45,700	1,600	
Vitamin A_2 (fraction rich in A_2)		max. 3450			(61, 62). 83% methyl alcohol soluble fraction from fish-liver oils.
Vitamin A_3 (fraction rich in A_3)		max. 2900			(63). 83% ethyl alcohol insoluble fraction from mammalian-liver oils.
"Sub vitamin A"		max. 3100			(64).
Vitamin D_2 mol. wt. 396	Hexane or ether	max. 2650	18,200	460	(75, 77, 78).
		min. 3100	0	0	
Vitamin D_3 mol. wt. 384	Ether	max. 2650	19,200	500	(75).
Vitamin D_4 mol. wt. 398	Ether	max. 2650	18,700	470	(75).
Vitamin E (α-tocopherol) mol. wt. 430	Isooctane	max. 2230	8,080	188	(79).
		min. 2550	300	7	
		max. 2980	3,140	73	
		min. 3160	0	0	
Vitamin K_1 mol. wt. 452	Hexane	max. 2400	16,000	355	(82, 83). For vitamin K_2 the positions of the maxima and minima are the same, but the ε values are slightly lower. See Fig. 12.
		min. 2410	15,600	344	
		max. 2430	18,700	412	
		min. 2460	16,100	355	
		max. 2490	20,000	440	
		min. 2550	12,800	282	
		max. 2600	18,000	398	
		min. 2655	15,000	330	
		max. 2700	18,000	398	
		min. 2870	700	15.5	
		max. 3250	3,200	70.8	

TABLE IV

Absorbing Characteristics of Fat-Soluble Provitamins

Substance	Solvent	Absorption Maxima and Minima			References and Remarks
		λ in A.	ε	$E^{1\%}_{1\,\text{cm.}}$	
α-Carotene mol. wt. 536	20% ether, 80% ethanol	max. 2470	23,900	420	(70). Maxima at 4480, 4790, and 5170 in CS_2 and at 2800, 3450, 4600 and 4920 in $CHCl_3$ (53). See references 53, 70, and 71 for additional literature.
		min. 3100	5,680	100	
		max. 4450	146,500	2,580	
		min. 4630	106,800	1,880	
		max. 4750	129,500	2,280	
		min. 5050	0	0	
β-Carotene mol. wt. 536	20% ether, 80% ethanol	max. 2500	21,400	400	(70).
		min. 3200	6,440	120	
		max. 4530	113,400	2,500	
		min. 4720	112,600	2,100	
		max. 4800	118,000	2,200	
		min. 5200	0	0	
	$CHCl_3$	max. 2800	20,100	385	(72). Maxima at 3180, 4500, 4855 and 5210 in CS_2 (53). See references 53, 71 and 74 for additional literature.
		min. 3200	6,970	130	
		max. 3500	15,510	289	
		min. 3625	12,000	224	
		max. 4350	94,400	1,764	
		min. 4450	93,200	1,730	
		max. 4620	101,400	1,892	
		min. 4800	91,200	1,700	
		max. 4920	94,300	1,760	
γ-Carotene mol. wt. 536	Hexane	max. 4075	11,500	229	(73). Maxima at 4675, 4950 and 5300 in CS_2 (53).
		min. 4125	11,000	205	
		max. 4350	23,900	429	
		min. 4400	22,500	420	
		max. 4600	34,000	634	
		min. 4800	23,000	429	
		max. 4900	28,500	516	
		min. 5250	0	0	
Ergosterol (provitamin D_2) mol. wt. 396	Ethanol	min. 2300	1,430	36.1	(76). The spectrum in isooctane is identical save for slightly lower extinction coefficients.
		max. 2620	6,940	175	
		min. 2630	6,850	173	
		max. 2710	10,000	253	
		min. 2755	8,580	217	
		max. 2820	10,600	268	
		min. 2890	5,450	138	
		max. 2930	6,060	153	
		min. 3175	35	0.8	
7-Dehydrocholesterol (provitamin D_3) mol. wt. 384	Ethanol	min. 2300	1,500	39.1	(76).
		max. 2625	7,400	193	
		max. 2710	10,400	271	
		min. 2760	8,830	230	
		max. 2815	10,750	280	
		min. 2880	5,530	144	
		max. 2930	6,150	160	
		min. 3210	36	0.9	

3. Precision of Measurement

The quantity $\log_{10}(I_0/I_x)$ in the Lambert-Beer law is known as the "density." Using "D" as a symbol for density, the Lambert-Beer expression may be written in the form

$$c = \frac{1}{kx} D \tag{8}$$

TABLE V

Absorbing Characteristics of Oestrone and Androsterone

Substance	Solvent	Absorption Maxima and Minima			References and Remarks
		λ in A.	ε	$E^{1\%}_{1\text{cm.}}$	
Oestrone mol. wt. 270	Ethanol	max. 2240	6,100	226	(76)
		min. 2485	222	8.2	
		max. 2810	2,045	75.8	
		min. 2860	1,910	70.7	
		max. 2870	1,940	71.8	
		min. 3100	0	0	
	Aqueous ethanol, neutral	min. 2450	429	15.9	(85). Oestradiol and oestriol have almost identical absorption and behave similarly toward pH change.
		max. 2800	2,273	84.2	
		min. 2950	0	0	
	Aqueous ethanol, *N*/3000 NaOH	min. 2025	570	21.1	
		max. 2800	2,025	75.0	
		min. 2950	0	0	
	Aqueous ethanol, *N*/100 NaOH	min. 2725	1,139	42.2	
		max. 2950	3,029	112.2	
		min. 3200	0	0	
Androsterone mol. wt. 290	Ethanol	max. 2925	42.6	1.47	(76)
		min. 2325	6.6	0.24	

The determination by the spectrophotometric method of the concentration of a known substance in a solution of unknown concentration involves the measurement of "D" (usually at the wave length at which "k" is a maximum) where both "k" and "x" are known. An error ΔD in the determination of the density then involves the following error in the estimation of the concentration

$$\Delta c = \frac{1}{kx} \Delta D \tag{9}$$

The fractional error $\Delta c/c$ in the estimation of concentration is equal to the fractional error $\Delta D/D$ in the determination of densities, for

$$\frac{\Delta c}{c} = \frac{\frac{\Delta D}{kx}}{\frac{D}{kx}} = \frac{\Delta D}{D} \tag{10}$$

Hence if the measuring method is such that the expected error ΔD remains constant independent of the value of D[5] (as is the case, for example, when match points in adjacent pairs of photographed spectra are determined visually) then it is advantageous that D be high in order to reduce the fractional error $\Delta c/c$. An increase in D, however, involves an increase in the concentration or cell length employed for measurement, either of which requires the use of additional material.

The precision with which D may be determined depends upon the spectrophotometric method employed and upon the skill and care of the investigator. The usual methods of spectrophotometry are described in detail elsewhere (16, 17). Of these, only two have been used extensively in the study of vitamins and hormones: (1) the photographic method, employing a sector or diaphragm type (*e.g.*, Spekker) photometer, in which the measurement of D involves the visual determination of positions of equal blackening, or "match points," on adjacent spectra photographed

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[5] The necessary condition for ΔD to be a constant independent of D may be seen by putting ΔD in differential form and equating to a constant, $-A$, *viz.*

$$dD = d \log_{10} \frac{I_0}{I} = -A$$

whence:

$$-\frac{dI}{I} = (\log_e 10)\,(-A)$$

$$\frac{dI}{I} = B, \quad \text{where} \quad B = A \log_e 10$$

Thus the condition is only fulfilled by a light-sensitive device in which the least difference in intensity, dI, which can be distinguished bears a constant ratio to the intensity I at which the measurement is made. This will be recognized as the Weber-Fechner law for visual intensity discrimination. However, the Weber-Fechner law does not hold for wide differences in intensity I. The justification for assuming its validity in spectrophotometric measurements lies in the fact that observations are usually made within a limited range of intensities within which the Weber-Fechner law holds to a fair degree of approximation. For example, in photographic spectrophotometry employing the visual matching of adjacent pairs of spectra, the exposure time is usually adjusted so as to obtain approximately the same degree of blackening of the plate for all spectrum pairs.

through the solvent and the solution; and (2) the photoelectric method, in which the transmission of the solution and solvent are measured alternately at a series of wave lengths by means of a photoelectric cell. Twyman and Lothian (18) have discussed the conditions for securing greatest accuracy in both of these methods. They state that in the photographic method with visual matching the eye can detect density differences in the photographic plate of about 0.06, which can be made to correspond to density differences, ΔD, in the absorbing solution of 0.01 if a high-contrast photographic plate is employed and developed to a contrast, or γ, of 6.[6] This represents about the limit of attainable accuracy, and seems to exceed considerably the precision to be expected in practice. Thus, von Halban and Eisanbrand (19) state that ΔD, the error in the estimation of D of the solution, can be limited to about 0.02 to 0.04, and Loofbourow (20) has found, in comparing determinations by various observers in his laboratory of the spectra of the same solutions, that it is unsafe to rely on a limitation of the overall error (including errors in the photometer and in its manipulation) to less than $\Delta D = \pm 0.03$. If one assumes an error of the latter magnitude in the determination of D, the fractional or percentage error may be computed from the value of D at which the measurements are made, since, as pointed out above, ΔD for visual measurements is independent of D. With the photometers usually employed in the photographic method, the highest value of D which can be measured conveniently is about 2.0. Since the solution (presumed to be of unknown concentration) must be adjusted in concentration by trial and error until its absorption maximum falls within the range of the instrument, it is most convenient to aim to employ solutions having densities of about 1.5 rather than higher values. Assuming, then, an average density value of 1.5 and a precision of measurement of D of ±0.03, the percentage accuracy to be expected in the determination of concentrations by the photographic method is

$$\frac{\pm \Delta c}{c} = \frac{\pm \Delta D}{D} = \frac{\pm 0.03}{1.5} = \pm 0.02 \equiv \pm 2\% \qquad (11)$$

In the photoelectric method, precision is limited by a minimum determinable difference, ΔI, in the light intensity rather than (as in the photographic method) by a minimum determinable density difference ΔD. The smallest value of ΔI which can be measured is, within the working range of the system, independent of I. The probable error, or probable deviation ±ΔI from the true intensity I, can therefore be expressed as a fraction of the full scale reading of the instrument (galvanometer, microammeter, potentiometer, etc.) from which the data are taken. Under the most ideal conditions, the probable error can hardly be expected to be less than ±0.1 per cent of full scale.[7] Under more usual conditions, as in the employment of photoelectric colorimeters, an error of ±0.5 per cent of full scale is more likely.

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[6] If ΔD is the minimum detectable density difference in the solution, γ is the contrast (*i.e.*, the ratio of change in density to change in log exposure) of the photographic plate and $\Delta D'$ is the minimum density difference detectable by the eye in examining the plate, then $\Delta D = \Delta D'/\gamma$.

It should be noted that the contrast of the photographic plate, γ, varies with the wave length, being in general considerably less at very short wave lengths (*e.g.*, in the range 1850 to 2300A.).

The relationship between ±Δc, the probable concentration error, and ±ΔI, the probable error in determining the light intensity, can be derived in various ways (18, 21). Perhaps the most straightforward is that of Twyman and Lothian (18), which is substantially as follows:

From the Lambert-Beer law:

$$kcx = D = \log_{10}\frac{I_0}{I_x} = \log_{10} I_0 - \log_{10} I_x \qquad (12)$$

If I_0 is constant, differentiation of D with regard to I yields

$$\frac{dD}{dI_x} = -\frac{\log_{10}e}{I_x} \qquad (13)$$

whence

$$\frac{dD}{D} = -\frac{\log_{10}e}{\log_{10}\frac{I_0}{I_x}} \cdot \frac{dI_x}{I_x} \qquad (14)$$

The fractional error in determining the concentration is, therefore, given by

$$\frac{\pm\Delta c}{c} = \frac{\pm\Delta D}{D} = \frac{0.4343}{D} \cdot \frac{\mp\Delta I_x}{I_x} \qquad (15)$$

This concentration error is least for a given intensity measurement error ΔI_x when the denominator on the right hand side of equation (14), $I_x\log_{10}(I_0/I_x)$, is a maximum. To determine the corresponding optimum density, the denominator can be differentiated with respect to I and equated to zero, thus:

$$\frac{d}{dI_x}\left[I_x \log_{10}\frac{I_0}{I_x}\right] = \log_{10}\frac{I_0}{I_x} - \log_{10}e = 0 \qquad (16)$$

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[7] Hogness, *et al.* (21) claim a precision of $\Delta I/I_0 = 0.002$ "under favorable working conditions" for their photoelectric spectrophotometer; that is an error of ±0.2 per cent. This is equivalent to an error of ±0.2 per cent of full scale if I_0 corresponds to full scale reading.

Whence:

$$\log_{10}\frac{I_0}{I_x} = \log_{10}e = 0.4343 \tag{17}$$

Hence the greatest precision is obtained when measurements are made with a combination of concentration and cell thickness such that $D = 0.4343$, which corresponds to $I_x = 0.368I_0$, $I_0 = 2.72I_x$.

Applying equation (15) and assuming that measurements are to be made at the optimum density, the concentration error becomes

$$\frac{\pm\Delta c}{c} = \frac{0.4343}{0.4343}\cdot\frac{\mp\Delta I_x}{I_x} = \frac{\mp\Delta I_x}{0.368\,I_0} \tag{18}$$

If I_0 corresponds to full scale readings, and if the probable error is ±0.5 per cent of full scale, then

$$\frac{\pm\Delta c}{c} = \frac{\mp 0.005I_0}{0.368I_0} = \mp 0.0136 \equiv \mp 1.36\% \tag{19}$$

An error of the order of ±1 per cent in the estimation of concentrations may therefore be expected with the photoelectric method of absorption spectrophotometry. These considerations apply as well to photoelectric colorimetry and they will be referred to again in discussing the precision obtainable with colorimetric techniques.

The amount of material necessary for the measurements by either the photographic or photoelectric techniques depends upon the shape of the cell used as well as upon the density value D at which measurements are made. In general, cells having face areas of the order of 1.5 sq. cm. must be employed to afford sufficient clearance for the light beam. To fill a cell of such face area requires about 2 ml. per cm. of cell length, when allowance is made for losses in manipulation, solution taken up by the cell arms (if any), etc. Using this value, the quantities of materials required for measurement may be calculated from the Lambert-Beer law as follows: $W = cV$, where "W" is the required weight of material in mg., "c" is the concentration in mg. per ml. and "V" is the cell volume in ml. $V = 2x$, where "x" is the cell length in cm. Combining these equations with equation (6) and solving for W,

$$W = \frac{2}{k}\log_{10}\frac{I_0}{I_x} = \frac{2D}{k} \tag{20}$$

in which "k" is in units of mg. per ml. concentration and cm. cell thickness. For the photographic method, $W = 3/k$ if a density of 1.5 is employed in the measurements, and for the photoelectric method, $W = 0.8/k$ using a density of 0.4.

Table VI is a compilation of (1) the concentrations of vitamins and hormones necessary for optimum spectrophotometric determinations and (2) the precision of concentration measurements obtainable, both as computed from the above considerations.

4. Absorption of Mixtures

The rules relating to the absorption of mixtures of substances are important in determining the relative concentrations of two or more substances in a solution and in compensating for the absorption of the solvent and for reflections occurring at the cell faces. If solutions of concentrations c_1, c_2, c_3, etc., and extinction coefficients k_1, k_2, k_3, etc. are placed one after another in the light path, and if I_0, I_{x_1}, I_{x_2}, $\cdots$ I_{x_n} represent respectively the intensity of the incident light and the intensities of the light transmitted by each succeeding solution, then

$$I_{x_1} = I_0 10^{-k_1c_1x_1},\ I_{x_2} = I_{x_1}10^{-k_2c_2x_2}\ ,\ \cdots\ I_{x_n} = I_{x_{n-1}}10^{-k_nc_nx_n} \qquad (21)$$

On combining these equations by the elimination of like terms, the following expression results:

$$I_{x_n} = I_0 10^{-k_1c_1x_1}10^{-k_2c_2x_2}\cdots 10^{-k_nc_nx_n} \qquad (22)$$

It is obvious from this expression that the sequence in which the solutions occur is immaterial; they may, in fact, be mixed together so that their molecules are distributed in random fashion one after the other without influencing the validity of the expression (provided there are no chemical interactions). Equation (22) may be written

$$\frac{I_{x_n}}{I_0} = 10^{-k_1c_1x_1-k_2c_2x_2\cdots-k_nc_nx_n} \qquad (23)$$

If the substances are together in a mixture, x is the same for all components. On simplification and solution for kc, equation (23) then reduces to

$$k_1c_1 + k_2c_2 + \cdots + k_nc_n = \frac{1}{x}\log_{10}\frac{I_0}{I_x} \qquad (24)$$

Equation (24) may be applied in numerous ways. As a first example, suppose that it is desired to determine the concentrations of each of two substances in a mixture. If c_1 and c_2 are the unknown concentrations of the two substances, k_1 and k_2 their extinction coefficients at a wave length λ, k_1' and k_2' their extinction coefficients at some other wave length λ', and D and D′ the densities, $\log_{10}(I_0/I_x)$, of the mixture at the two wave lengths, then from (24)

$$k_1c_1 + k_2c_2 = \frac{1}{x}D; \qquad k_1'c_1 + k_2'c_2 = \frac{1}{x}D' \qquad (25)$$

whence

$$c_1 = \frac{k_2 D' - k_2' D}{x(k_1' k_2 - k_1 k_2')} \qquad c_2 = \frac{k_1 D' - k_1' D}{x(k_1 k_2' - k_1' k_2)} \tag{26}$$

If the densities D and D′ are measured experimentally and the extinction coefficients of the constituent substance are known at the appropriate wave lengths, the concentrations of the substances may be computed from (26). For greatest precision, the two wave lengths must be chosen so that the absolute value of $(k_1 - k_1' - k_2 + k_2')$ is as great as possible.[8] The setting up of a number of simultaneous equations analogous to (24) and equal in number to the number of unknowns and the solving of these equations for the unknowns can be employed in a variety of ways. Twyman and Allsopp (22) describe the extension of the method to mixtures of three components and other problems. Of particular interest in the vitamin field is the application by Reerink and van Wijk (23) of analogous simultaneous equations in demonstrating that the nature of the photochemical transformation products obtained when ergosterol is irradiated with ultraviolet light depends upon the wave lengths used for irradiation.

As a second example, suppose that it is desired to eliminate from the measurements the effects of absorption of the solvent and of reflections from the cell faces. This may be done by comparing the intensity "I_x" of the light transmitted by a cell containing the solution with the intensity "I_0'" of light transmitted by an identical cell containing the solvent alone. For if "I_0" is the incident intensity, "A" the fraction of light transmitted as a result of reflection losses at the entering cell surfaces, "B" the fraction of light transmitted as a result of reflection losses at the exit cell surfaces, "α" the extinction coefficient of the solvent, "k" and "c" the extinction coefficient and concentration of the solute, and "x" the length of the light path in the cell, then from equation (23)

$$I_x = I_0 A 10^{-x(\alpha + kc)} B \tag{27}$$

and

$$I_0' = I_0 A 10^{-x\alpha} B \tag{28}$$

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[8] This may be demonstrated as follows: Given a solution of total concentration $c = c_1 + c_2$ the absorption of which is to be measured in a cell of length x, let it be required to choose the wave lengths (in terms of related extinction coefficients) at which to measure the densities D and D′ such that the error $\pm\Delta c_1$ in the determination of c_1 will be as small as possible for a given error $\pm\Delta(D - D')$ in the determination of the densities. Upon substituting $c_2 = c - c_1$ in equations (25), subtracting the second equation from the first, and solving for c_1, the following equation results: $c_1 = [(1/x)(D - D') - c(k_2 - k_2')]/(k_1 - k_2 - k_1' + k_2')$. Since the second numerator term is constant, $\pm\Delta c_1 = \pm\Delta(D - D')/x(k_1 - k_1' - k_2 + k_2')$. Hence the error $\pm\Delta c_1$ is a minimum for a given error $\pm\Delta(D - D_2')$ when the quantity $(k_1 - k_1' - k_2 + k_2')$ is a maximum.

Dividing (27) by (28),

$$\frac{I_x}{I_0'} = \frac{I_0 AB 10^{-\alpha x - kcx}}{I_0 AB 10^{-\alpha x}} \tag{29}$$

On simplification and on taking the logs of both sides, equation (29) reduces to the familiar Lambert-Beer expression (except that I_0' occurs in place of I_0) from which the coefficients arising from reflections and from absorption of the solvent have been eliminated, *viz.*

$$\log_{10} \frac{I_0'}{I_x} = kcx \tag{30}$$

This is the usual method of compensating for solvent and reflection effects in the absorption spectrophotometry of vitamins and hormones.

As a third example, suppose it is desired to compute the absorption of a complex molecule from the known absorptions of sub-molecular units of which it is composed. A specific example considered in a later paragraph is the absorption of thiamine as computed from the known absorptions of its thiazole and pyrimidine rings. In such a case, the molecule and its sub-units are all present in equi-molar concentrations. Hence if ϵ_1, ϵ_2, ϵ_3, etc. are the molecular extinction coefficients of the sub-units, "c" is the molar concentration of the molecule (and hence of its sub-units), and "ϵ" is the molecular extinction coefficient of the molecule, then from (24)

$$\epsilon_1 c + \epsilon_2 c + \cdots + \epsilon_n c = \frac{1}{x} \log_{10} \frac{I_0}{I_x} \tag{31}$$

but

$$\epsilon c = \frac{1}{x} \log_{10} \frac{I_0}{I_x} \tag{32}$$

whence

$$\epsilon = \epsilon_1 + \epsilon_2 + \cdots + \epsilon_n \tag{33}$$

This simple relationship is quite useful, as will appear from the subsequent discussion. The extension to more complex cases in which certain sub-units occur more than once in the molecule can be made, obviously, by the introduction of the proper integral coefficients in (33). It should be emphasized that in the derivation of (33) it was assumed that the bonding together of the sub-units did not influence their individual contributions to the absorption, an assumption which is by no means universally true.

5. Absorption Spectra of Individual Water-Soluble Vitamins

Vitamin B_1 (Thiamine, Aneurin). Early reports of the spectrum of thiamine were in disagreement, it being variously stated that the spectrum was characterized by a single marked maximum at 2600 Å (24), a single maximum at 2450 Å (25), two maxima at 2390 and 2700 Å (26), or two maxima at 2350 and 2650 Å (27). It was suggested that the differences might be attributable to the use of alcohol as a solvent by some workers (24, 25) and water by others (26, 27), but investigations by Wintersteiner, *et al.* (28) of several crystalline preparations showed them to exhibit maxima at 2350 and 2670 Å in either alcohol or water solution. The

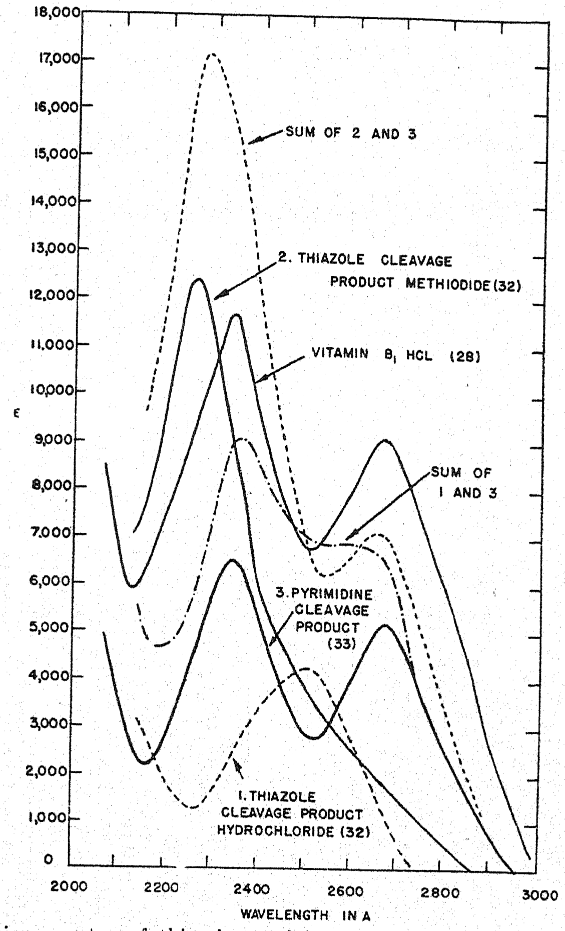

Fig. 2. Absorption spectra of thiamine and its pyrimidine and thiazole cleavage products. References in parentheses

single band at 2600 Å was found, later, to be in error (29). The single maximum at 2450 Å proved to be characteristic of acid solutions of thiamine and the two maxima at 2350 and 2650 Å of neutral or alkaline solutions (30). As has been suggested from the fact that the nitrogen of the thiazole ring is quaternary when linked to pyrimidine (31), the spectrum of thiamine is more closely represented by a summation of the spectrum of the methiodide of the thiazole cleavage product (32) with that of the pyrimidine cleavage product (33) than by a summation of the latter with the spectrum of the hydrochloride of the thiazole cleavage product (32) (Fig. 2). Changes of the absorption of various 6-amino pyrimidines as a function of pH are qualitatively analogous to those observed in thiamine (34, 35). This has led to the conclusion that the effect of pH on thiamine is entirely attributable to its influence on the pyrimidine ring (35, 36), but the data presented do not support this hypothesis quantitatively.

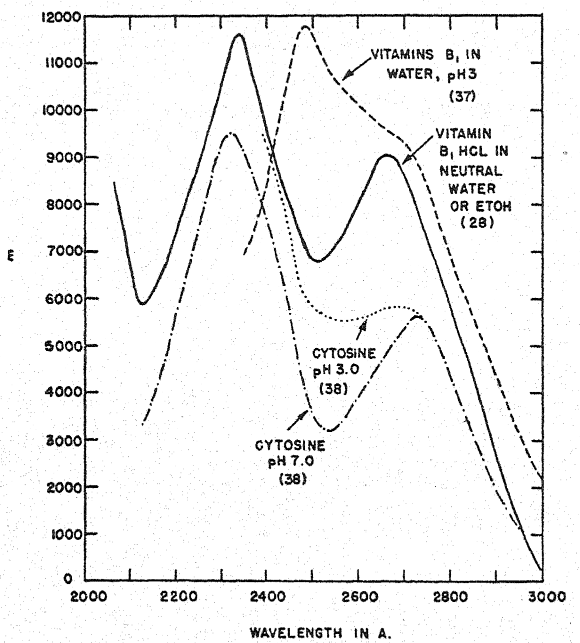

Fig. 3. Absorption spectra of cytosine and of thiamine in acid and neutral solution. References in parentheses

Fig. 3 shows the spectrum of thiamine in neutral alcohol or water solution according to Wintersteiner, *et al.* (28) and in water at pH 3.0 according to Ogsten and Peters (37), together with the spectrum of cytosine (38) which, of various naturally-occurring pyrimidines, most closely resembles thiamine in its absorption in neutral solution. Cytosine can be differentiated easily on the basis of pH effects (Fig. 3). Various naturally-occurring purines (guanine, uric acid, hypoxanthine) the spectra of which respond to pH much as does that of thiamine, have absorption maxima at wave lengths sufficiently different from those characteristic of the vitamin to permit them to be distinguished easily from thiamine. Published values of extinction coefficients for thiamine show considerable variation.

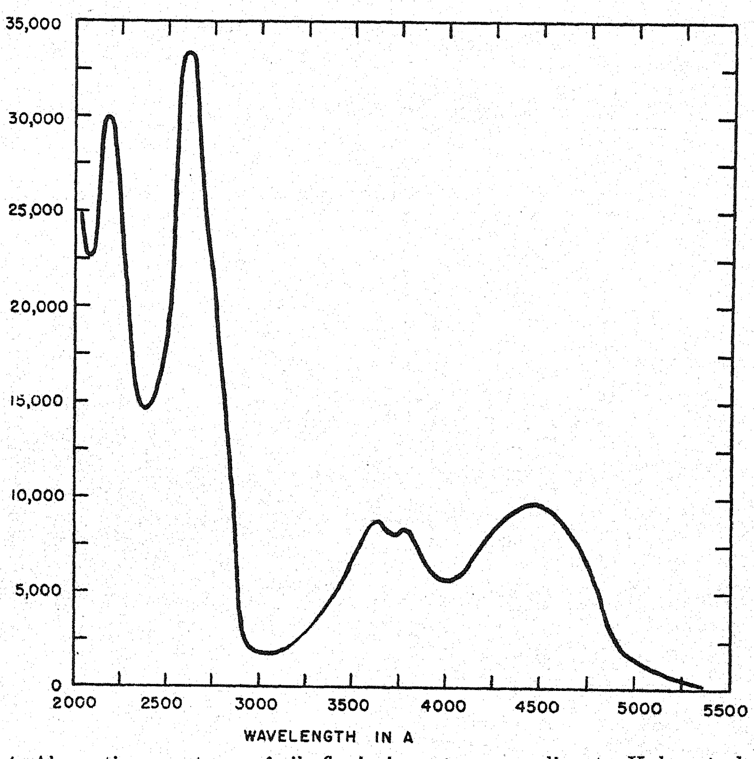

Fig. 4. Absorption spectrum of riboflavin in water, according to Kuhn, *et al.* (41)

The data of Wintersteiner, *et al.* (28), Melnick (39), and Ogsten and Peters (37) have been used in the summary tables (Tables II and VI).

Vitamin B_2 (*Riboflavin*). The absorption spectrum of riboflavin (lactoflavin) in common with that of other flavin complexes, is characterized by marked maxima near 2200 and 2700 Å, together with less pronounced maxima near 3700 and 4500 Å (40, 41, 42). The absorption is due to the alloxazine nucleus (43). In nearly neutral water solution, the precise position of the bands is 2200, 2650, 3650, and 4450 Å (41) (Fig. 4). The spectrum is not markedly influenced by pH, the principal effect being a slight shifting of the 3650 and 4450 Å bands toward longer wave lengths with increase in pH in the alkaline range (44). Lumilactoflavin, the photo-oxidation product (soluble in $CHCl_3$), has a similar spectrum (40).

Vitamin B_6 (Pyridoxin). The spectrum of pyridoxin has been reported by several workers (45, 46, 47). In nearly-neutral water solution it exhibits two maxima, near 2550 and 3260 Å (47). In strongly-acid solution (ca. pH 2) these bands no longer appear, but there is a marked maximum at about 2910Å (47). Spectra of the vitamin according to Stiller, *et al.* (47) for a range of pH from 2.1 to 10.2 are shown in Fig. 5. The complex and characteristic effects of pH on the spectrum of pyridoxin should serve to distinguish it readily from other naturally-occurring substances.

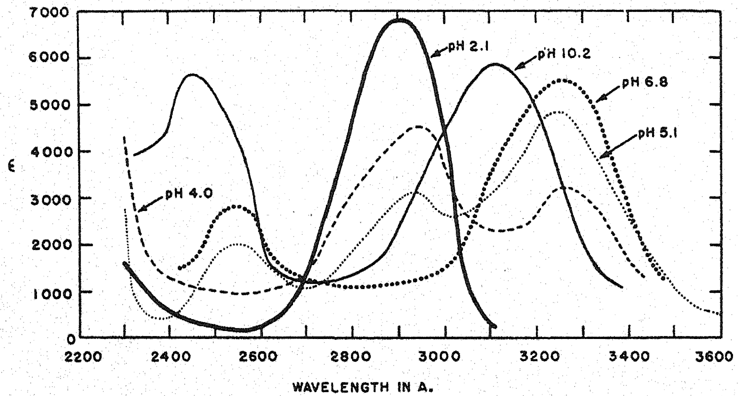

Fig. 5. Absorption spectra of pyridoxin in water at various pH's, according to Stiller, *et al.* (47)

Vitamin C (Ascorbic Acid). Ascorbic acid (hexuronic acid) was first reported to be characterized by a single absorption band at 2650 Å (48). On more careful investigation (49, 50, 51) it developed that the position of the maximum is a function of the solvent, pH, and concentration (Fig. 6). At low concentrations (up to about 25 mg. per l.) in water at neutral pH or in methyl alcohol the maximum is at 2630–2650 Å for fresh solutions. In ethyl alcohol, at high concentrations (ca. 10,000 mg. per l.) in water, and at 20 mg. per l. in water acidified to pH 3, the maximum for fresh solutions is at 2450 Å. In all of these instances, the extinction coefficient is about the same ($E^{1\%}_{1cm.} = 550$). The deviations from Beer's law are marked, apparently because of the shift in absorption maximum with concentration, but they are insufficient to cause appreciable error in the measurement of concentrations less than about 25 mg. per l. Departure from Beer's law is said to be especially marked in methyl alcohol (50).

An additional difficulty is encountered in the quantitative determination of the spectrum of this substance because of the rapid degradation of the absorption maximum which accompanies oxidation of the vitamin in solution. This effect is less marked in acid solutions. It may be suppressed almost entirely by the addition to solutions buffered to pH 5 of KCN at a concentration one-tenth that of the ascorbic acid (52, 53). The oxidation is accelerated by the presence of copper ions or other heavy metal ions in the solution, and these are bound by the KCN, but there appears to be, in addition, a direct action of KCN on the vitamin itself (52). In the spectrophotometric assay for ascorbic acid of materials (*e.g.*, fruit juices) containing other substances, the band due to the vitamin may be degraded in an aliquot of the sample by addition of copper sulfate solution and alkali, and this aliquot may then be used as a blank (53). Thus the lability of the vitamin may be made use of in distinguishing its absorption from that

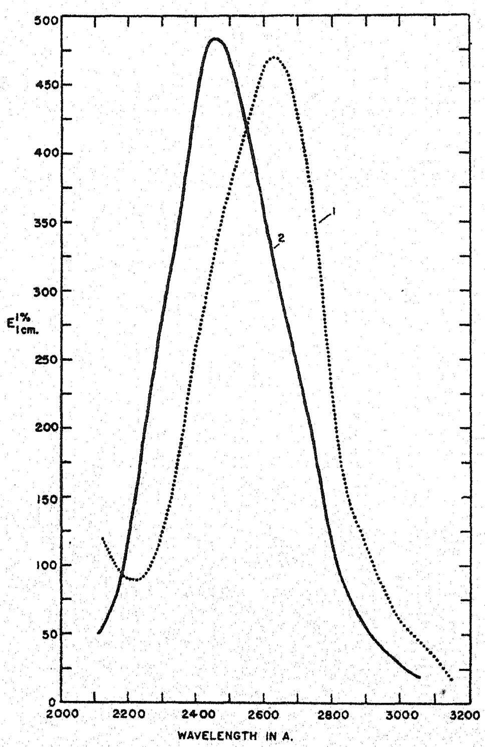

Fig. 6. Absorption spectra of *l*-ascorbic acid, according to Herbert, Hirst, *et al.* (50) Curve 1, ascorbic acid or its sodium salt in water at 20 mg. per l. Curve 2, ascorbic acid in alcohol or acid water at 20 mg. per l. Note that the extinction coefficients above are lower than the more recent values listed in Table II.

of other naturally-occurring substances having similar absorbing characteristics.

Other Water-Soluble Vitamins. Nicotinic acid and nicotinamide have a single marked maximum at 2600 Å (54) (Fig. 7). Their spectra are similar to that of adenine (55), but the extinction coefficient of adenine at the 2600 Å maximum is about three times as great. Biotin (56) and folic acid (57) have negligible absorption throughout the visible and ultraviolet regions of the spectrum. The spectrum of pantothenic acid appears not to have been reported, but from its chemical structure it would be expected to show little if any absorption in the range of wave lengths usually investigated.

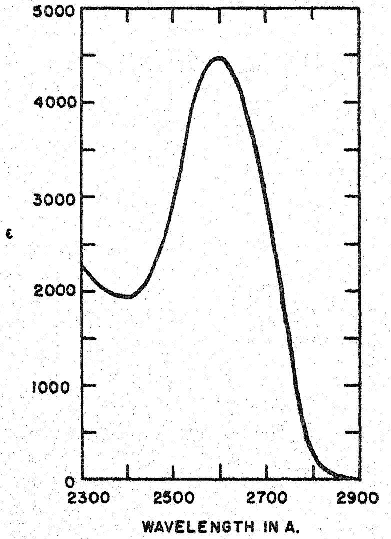

Fig. 7. Absorption spectrum of nicotinic acid amide in water, according to Warburg, *et al.* (54)

6. *Absorption Spectra of Individual Fat-Soluble Vitamins*

Vitamin A and Its Precursors. Vitamin A_1 is characterized by a single broad band[9] with its maximum at 3280 Å (58, 59, 60) (Table III). Vitamin A_2 has similar absorption, but with the maximum displaced to 3450–3500 Å (61, 62). A third substance, A_3, with vitamin A activity and with an absorption maximum at 2850–2900 Å has been found in mammalian

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[9] This applies to measurements in solution at room temperature. As in the case of many other substances, additional fine structure appears if the measurements are made at very low temperatures, such as those of liquid air. Maxima at 3350, 2900, 2770, 2580, and 2510 Å have been reported for such measurements (53).

(63) and other liver oils (61, 62). Recently the occurrence has been reported (64) of a fourth substance, "sub vitamin A," with certain properties characteristic of A vitamins and with an absorption maximum at 3100 Å. Typical spectra of the A vitamins are shown in Fig. 8. A method of determining the adulteration of halibut and cod liver oils with mammalian liver oils (*e.g.*, whale liver oil) is based on the presence of the band near 2900 Å in the latter but not in the former (65).

Spectrophotometry has been used more extensively as an assay method for vitamin A than for any other vitamin. The usual procedure is to determine the absorption at 3280 Å as an indication of the A_1 content of

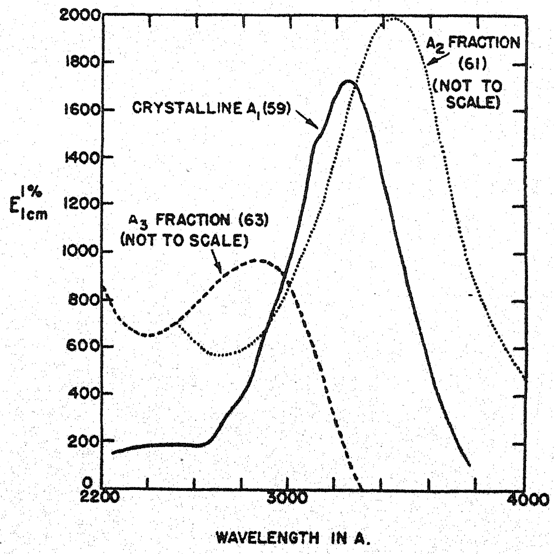

Fig. 8. Absorption spectra of crystalline vitamin A_1 in ethanol and of liver-oil fractions rich in A_2 and A_3. References in parentheses

the material to be assayed. Various absorptiometers, or ultraviolet "colorimeters," have been devised especially for this purpose (66). The absorption of interfering substances (in particular, unsaturated fatty acids) is not of great significance in oils of high activity (*e.g.*, halibut liver oils) which exhibit an extinction at 3280 Å of $E^{1\%}_{1\text{ cm.}} > 7$, but for precise assays of oils with lower 3280 Å extinction, it is necessary to eliminate the effects of interfering substances by measuring the absorption of the unsaponifiable fraction (53). Since saponification is not a positive method for removing all interfering substances, it has been suggested (67) that the following criteria be used to determine the "normality" of vitamin A preparations: (1) the absence of fine structure in the absorption spectrum (indicating cyclized vitamin A); and (2) a well-defined 6170 Å maximum in the antimony trichloride test, with $E^{1\%}_{1\,\text{cm.}}$ at 617 approximately twice that at 580 mμ. There is considerable controversy about the value of the constant "C" which should be used in the following equation for converting the absorption at 3280 Å to international biological units, "IU":

$$C \times E^{1\%}_{1\,\text{cm.}} = \text{IU per g.} \tag{34}$$

English workers favor a conversion factor of about 1600, whereas American workers find values ranging from 2150 to 2700 (see Coy, *et al.* (68)). These discrepancies are discussed at length in a recent review by Morton (69).

The carotenoid precursors of vitamin A have spectra consisting of three or more maxima in the visible and ultraviolet regions (70–74). The number and positions of the maxima depend upon the particular carotene isomer (α, β, or γ) and the solvent. Typical spectra are shown in Fig. 9, and data for the absorption maxima and minima are recorded in Table IV.

Vitamin D and Its Precursors. The absorption spectra of the various vitamin D precursors and their photochemical transformation products have been reported in numerous papers concerning the chemistry and photochemistry of vitamin D. The earlier literature has been reviewed in detail with specific reference to the rôle of absorption spectrophotometry in these investigations (1, 74). Brockmann's review (75) of the chemistry of vitamin D contains references to later work.

The vitamin D precursors, ergosterol (provitamin D_2), 7-dehydrocholesterol (provitamin D_3), 22-dihydroergosterol (provitamin D_4), 7-dehydrositosterol and 7-dehydrostigmasterol have closely similar spectra, with four principal maxima at about 2625, 2710, 2815, and 2930 Å (75–76). Data are shown in Fig. 10 and Table IV for ergosterol and 7-dehydrocholesterol in ethyl alcohol.

Vitamins D_2, D_3, and D_4 are likewise similar in absorption, each being characterized by a single broad band with a maximum at about 2650 Å. The molecular extinction coefficients are slightly different, being highest for D_3 (Table III) (75, 77, 78). The necessity of avoiding photochemical degradation of the vitamin in obtaining spectra of vitamin D has been emphasized (77). Of intermediate and end-products of importance in the photochemistry of vitamin D, lumisterol has two maxima at 2650 and 2800 Å, takysterol a single maximum at 2800 Å, toxisterol a single maximum at 2480 Å, and suprasterol only end absorption (1, 75). Vitamin D_1, being a mixture of D_2 and lumisterol, has a spectrum intermediate between these two. The extinction coefficients of the mixture (D_1) are related to those of its constituents in accordance with equation (25).

Vitamin E (α-Tocopherol). Of substances possessing vitamin E activity,

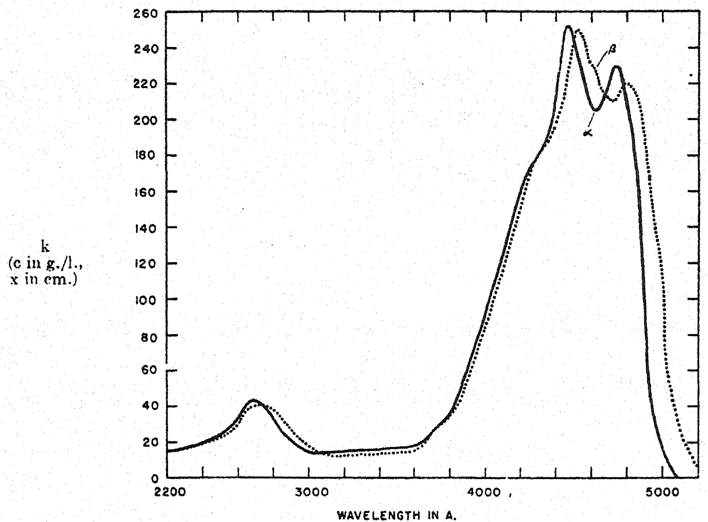

Fig. 9. Absorption spectra of α- and β-carotene in 20% ether, 80% ethanol, according to Miller (70)

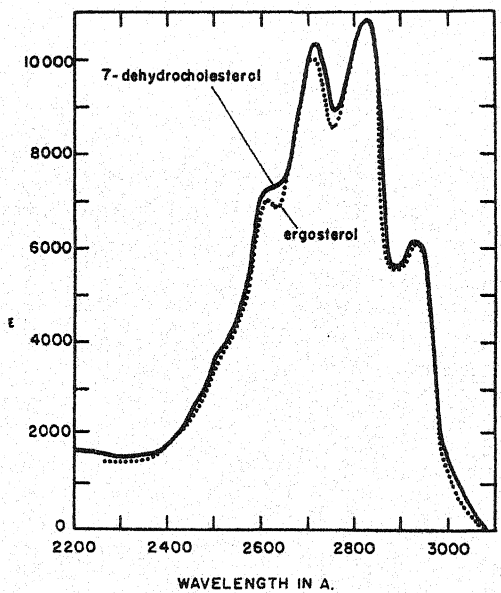

Fig. 10. Absorption spectra of 7-dehydrocholesterol and ergosterol in ethanol, according to Hogness, *et al.* (76)

α-tocopherol is the most potent. Its absorption is characterized by a broad maximum at about 2980 Å, a minimum at about 2550 Å, and a narrow, highly absorbing band at about 2250 Å (79) (Fig. 11). Spectro-

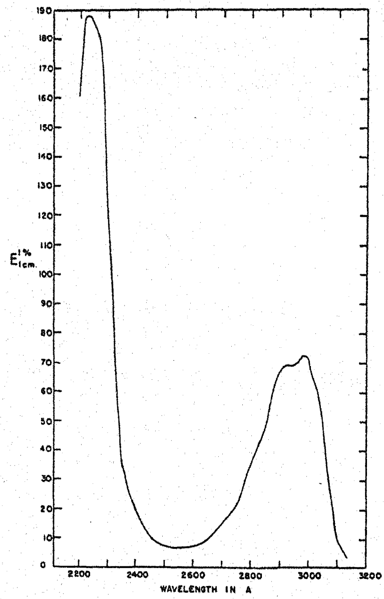

Fig. 11. Absorption spectrum of α-tocopherol in isooctane, according to Emerson, *et al.* (79)

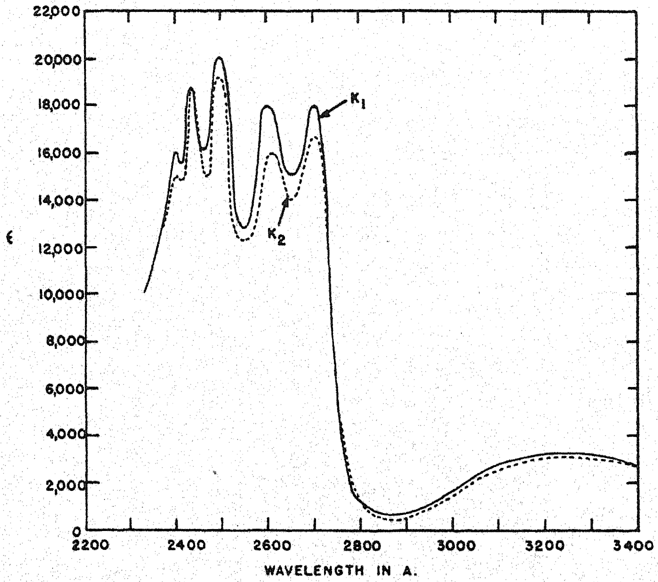

Fig. 12. Absorption spectra of vitamins K_1 and K_2 in hexane, from data of Ewing as reported by Doisy, *et al.* (82)

scopic methods have been used as a means of tracing the disposition of α-tocopherol in animals (80). β-Tocopherol has similar absorption (81).

Vitamin K. The naphthoquinone ring of substances having K activity results in their exhibiting complex selective absorption consisting of several maxima and minima in the ultraviolet region of the spectrum beyond 2800 Å (82, 83). Spectra of vitamin K_1 and K_2 are shown in Fig. 12, and data are recorded in Table III.

7. *Absorption Spectra of Selected Hormones and Enzymes*

Hormones. Since only steroid hormones have been studied extensively by absorption spectrophotometry, they alone will be considered here. References to such spectroscopic data as are available regarding other hormones will be found in Morton's review (53). Oestrone (theelin), oestradiol, and oestriol in acid or neutral aqueous alcohol solution are characterized by a broad band with its maximum at about 2800 Å (84, 85), and a somewhat more pronounced maximum at about 2240 Å (76). In alkaline solutions, the 2800 Å band shifts to 2950–3000 Å (84, 85). In the case of oestrone, the shift has been attributed to enolization of the 17-keto group (84). Callow (85) states, however, that the shift is more likely attributable to salt formation by the phenolic 3-hydroxy group, since oestradiol and oestriol, in which tautomerism of the type suggested cannot take place, both exhibit the change, whereas oestrone methyl ether, in which the possibility of salt formation is blocked, does not show it.

Androsterone has a broad absorption band with its maximum at 2925 Å. Androstenedione has a similar broad band with its maximum at 2408 Å (76). For the spectra of certain related sterols, see reference (76).

Enzymes. Because of their close relationship to vitamins of the B complex, certain enzymes and coenzymes should be mentioned. Diphospho- and triphosphopyridine nucleotides (coenzymes 1 and 2) owe their absorption to the nicotinic acid and adenine portions of the molecule (54, 86), the carbohydrate and phosphoric acid contributing inappreciably, as would be expected. Since both adenine (54, 55) and nicotinic acid (54) have a single maximum at 2600 Å, coenzymes 1 and 2 afford a simple test of the validity of equation (33), according to which the molecular extinction coefficient of the coenzyme should equal the sums of the molecular extinction coefficients of adenine and nicotinic acid. That this is closely true may be seen from the following, in which the subscripts "a," "n," and "c" denote adenine, nicotinic acid, and coenzyme 2, respectively:

From reference 54, 55:

$$\epsilon_a + \epsilon_n = 12{,}500 + 4{,}460 = 16{,}960$$

From reference 54:

$$\epsilon_c = 16{,}650$$

TABLE VI

Assay of Vitamins and Hormones by Absorption Spectrophotometry

Concentrations shown are for 1 cm. cells. Required concentrations for other cell lengths may be computed by dividing the values shown by the cell length in cm. About 2 ml. of solution are required per cm. of cell length. Probable errors have been computed as discussed in the text.

Substance	Solvent and pH	λ, A.	$E^{1\%}_{1cm}$	Photographic Method D = 1.5, 1 cm. cell		Photoelectric Method D = 0.434, 1 cm. cell	
				Required c, μg./ml.	Probable error, μg./ml.	Required c, μg./ml.	Probable error, μg./ml.
Thiamine (B_1)	Neutral water or ethanol	2,670	257	5.81	±0.12	1.68	±0.017
Riboflavin (B_2)	Water	2,650	929	1.61	±0.032	0.47	±0.0047
Pyridoxin (B_6)	Water, pH 2.1	2,910	402	3.73	±0.074	1.08	±0.011
	Water, pH 6.8	3,260	326	4.60	±0.092	1.33	±0.013
l-Ascorbic acid (C)	Water, pH < 3	2,450	550	2.73	±0.055	0.79	±0.0079
Nicotinamide	Water	2,600	417	3.60	±0.072	1.04	±0.010
Vitamin A_1	Ethanol	3,280	1,720	0.872	±0.017	0.247	±0.0025
Vitamin D_2	Hexane or ether	2,650	460	3.26	±0.065	0.944	±0.0094
Vitamin D_3	Ether	2,650	500	3.00	±0.060	0.868	±0.0087
Vitamin E	Isooctane	2,980	73	20.5	±0.41	5.94	±0.059
Vitamin K_1	Hexane	2,490	44	34.1	±0.68	9.88	±0.099
α-Carotene	Ether-ethanol	4,450	2,580	0.581	±0.012	0.168	±0.0017
β-Carotene	Ether-ethanol	4,800	2,200	0.682	±0.014	0.195	±0.0020
γ-Carotene	Hexane	4,600	634	2.37	±0.047	0.686	±0.0069
Ergosterol	Ethanol	2,820	268	5.60	±0.112	1.62	±0.016
7-Dehydrocholesterol	Ethanol	2,815	280	5.36	±0.107	1.55	±0.016
Oestrone	Aqueous ethanol	2,800	84.2	17.8	±0.356	5.16	±0.052
Androsterone	Ethanol	2,925	1.47	102.0	±2.04	29.6	±0.30

Analogously, the spectra of flavin-adenine dinucleotide, flavoprotein enzymes, pyridinoprotein enzymes, cocarboxylase, thiaminoprotein enzymes, etc., are equivalent to the summation curves of the spectra of their absorbing constituents plotted on a molecular extinction coefficient basis, the absorbing constituents being adenine, flavins, pyridine structures, and thiamine on the one hand, the spectra of which have been discussed, and proteins (characterized generally by an absorption maximum at 2800 Å) on the other. Literature regarding the spectra of these substances has been reviewed elsewhere (see, for example, Green (87) and Hogness (88)).

As linked together in coenzyme molecules, the absorbing groups exhibit characteristic absorption changes on oxidation and reduction. Thus diphosphopyridine nucleotide in the reduced form has a band at 3450 Å which does not appear in the oxidized coenzyme. Analogous changes take place in the methiodide of nicotinic acid upon oxidation and reduction.

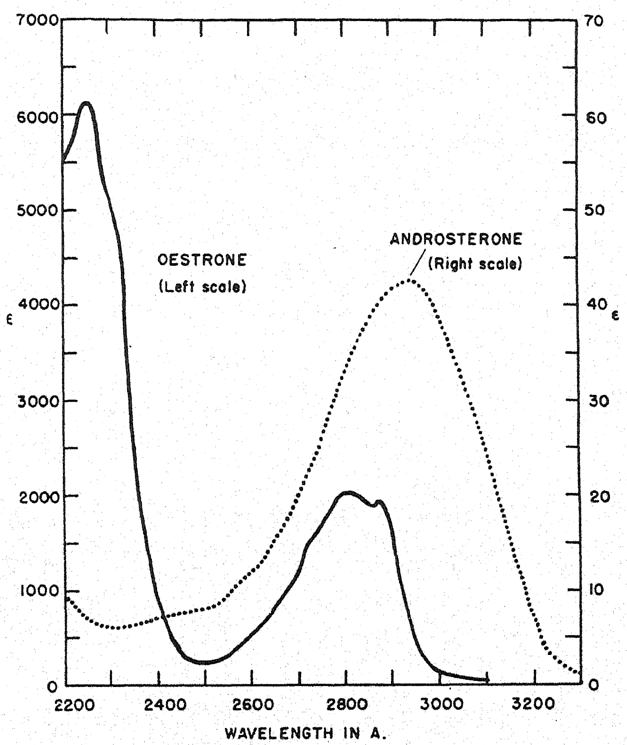

Fig. 13. Absorption spectra of oestrone and androsterone in ethanol, according to Hogness, *et al.* (76)

These relationships of absorption to oxidation-reduction are useful in the identification and quantitative determination of coenzymes (87, 88).

III. Colorimetry

1. *Methods and Precision of Measurement*

Colorimetry in the restricted sense defined in Section I, 2 (otherwise known as "absorptiometry") presupposes the use of some means, such as filters or a simple monochromator, for isolating a narrow region of the spectrum. The spectral region transmitted by the filter or monochromator is chosen to coincide with the absorption maximum of the substance of which the concentration is to be measured.[10] On the one hand, filters have the advantage of simplicity and economy. On the other hand, monochromators afford better isolation of the desired spectral region and permit rapid determination of the absorption curve of the substance in question. From the absorption curve, selection of the optimum wave band for the measurements may be made easily and quickly. Good isolation of narrow spectral regions may be obtained by the use of filters in combination with a light source, such as a mercury arc, which emits a spectrum of widely-separated lines. Resolution is gained in this way only at the expense of flexibility, however, since only those wave lengths can then be employed which correspond to the spectrum lines of the source.

Given a source of light and a system (monochromator or filters) for isolating the spectral region to be employed in the measurements, the problem of absorptiometry then becomes one of comparing the intensity, "I_x," of monochromatic light transmitted by a solution with that, "I_0," transmitted by a corresponding blank. The familiar Lambert-Beer law may be applied, *viz.*,

$$kcx = \log_{10} (I_0/I_x) \text{ (equation 6).}$$

The extent to which this law is applicable depends upon how nearly monochromatic is the wave band employed in the measurements and upon how closely Beer's law is approximated.

As in the case of absorption spectrophotometry, the value of I_0/I_x may be determined either (a) by the visual matching of intensities, adjusted for solvent and solution by some convenient means such as crossed polarizing prisms, or (b) by photoelectric methods. With the first technique, (a), precision is limited to

$$\pm\Delta c/c = \pm\Delta D/D \text{ (from equation 10),}$$

where "c" is the concentration measured, "D" the corresponding optical density, $\log_{10} (I_0/I_x)$, of the solution, and $\pm\Delta D$ the error in visual density matching (of the order of ±0.05 to ±0.1, depending upon the observer, the color matched, etc.). Further error is introduced by the limit of accuracy with which the scale of the instrument can be read and by the absolute accuracy error of the instrument itself, but the visual matching error is the factor which ultimately limits the precision obtainable.

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[10] This substance is in general a colored complex formed by the coupling of the substance to be assayed with a suitable reagent capable of combining with it to form a colored compound.

The photoelectric method, (b), is now almost universally used, and is therefore of greater interest. The limiting precision obtainable with it is, from equation (15)

$$\frac{\pm\Delta c}{c} = \frac{0.4343}{D} \times \frac{\mp\Delta I_x}{I_x} = B\,\frac{\mp\Delta I_x}{I_0} \tag{35}$$

$$\text{where } B = \frac{0.4343}{D} \times \frac{I_0}{I_x}$$

TABLE VII

Relation of Concentration Error to Per Cent Transmission in Photoelectric Colorimetry

D	Per Cent Transmission $\left(100\,\frac{I_x}{I_0}\right)$	B [Equation (35)]	Per Cent Concentration Error $100\left(\frac{\pm\Delta c}{c}\right)$	
			$\frac{\pm\Delta I}{I_0} = \pm0.005$*	$\frac{\pm\Delta I}{I_0} = \pm0.001$†
α	0	—	—	—
1	10	434.3	±2.17	±0.43
0.6990	20	310.7	±1.55	±0.31
0.5228	30	273.8	±1.37	±0.27
0.4343	36.8	271.8	±1.36	±0.27
0.3979	40	275.4	±1.38	±0.28
0.3010	50	288.5	±1.44	±0.29
0.2217	60	326.5	±1.63	±0.33
0.1596	70	388.7	±1.94	±0.39
0.0969	80	560.2	±2.80	±0.56
0.0457	90	980.9	±4.90	±0.98
0	100	α	—	—

* Corresponding to an error of ±0.5 scale division if I_0 corresponds to scale reading of 100.

† Corresponding to an error of ±0.1 scale division if I_0 corresponds to scale reading of 100.

As has been shown in the case of absorption spectrophotometry (equations 16 and 17) the least error occurs when D = 0.4343, corresponding to 36.8 per cent transmission. Table VII illustrates the manner in which the error varies with "D" and with per cent transmission. Within the range of approximately 20 per cent to 60 per cent transmission, the error is reasonably close to the minimum value. Hence for greatest precision, concentrations should be kept within the limits corresponding to this range of transmission values.

Were it customary for investigators using colorimetric procedures to measure the extinction coefficients "k" of the absorbing complexes with

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which they are dealing, it would be possible to compute from such data the required concentrations of materials for optimum accuracy of measurement and the probable concentration error for an error $\pm\Delta(I_x/I_0)$ in the intensity measurements. Unfortunately, however, extinction coefficients are seldom given in the literature.

The usual practice in colorimetry is to determine a calibration curve for the instrument in terms of concentration "c" and logs of readings "G":

$$c \propto (\log_{10} G_0 - \log_{10} G_x) \qquad (36)$$

in which "G_0" and "G_x" are the galvanometer (or other indicating device) readings obtained through the blank and through the solution, respectively. If, as is customary, the instrument is operated in such a manner that G_0, the measurement obtained on the blank, is 100 on the indicator scale and G_α, corresponding to no light transmission, is 0 on the same scale, then equation (36) reduces to

$$c \propto (\log_{10} 100 - \log_{10} G) \propto (2 - \log_{10} G) \qquad (37)$$

The symbol "L" is frequently used (89) for the quantity $(2 - \log_{10} G)$, whence

$$c \propto L \qquad (38)$$

Two advantages of using a calibration curve are that (1) linearity of response and (2) validity of Beer's law are not assumed. The use of a new calibration curve, made with a standard solution of the substance, for each set of measurements is of use in eliminating errors due to changes in the reagents or to variations which may occur from time to time in the manipulations. If the calibration curve of c versus L is a straight line over a wide range of concentrations, then $Ac = L = \log_{10} (G_0/G_x)$. This is equivalent to the Lambert-Beer law (equation 6) with $A = kx$ and $(G_0/G_x) = (I_0/I_x)$, and implies: (a) that the response of the instrument is a linear function of the transmitted light intensity, (b) that Beer's law holds, and (c) that the light employed is approximately monochromatic.

The photocell circuits employed in colorimetric measurements and other aspects of instrumentation have been reviewed in detail elsewhere (90–93).

2. *Applications*

Colorimetry has such broad application in the assay of vitamins and hormones that the literature regarding colorimetric assay methods is quite extensive. The majority of papers deal with the important problems of the elimination of interfering substances and the improvement of the specificity of color reactions. Recent work has been reviewed by György

(94) for water-soluble vitamins, by Morton (69) for fat-soluble vitamins, and by Pfiffner and Kamm (95) for hormones. No attempt will be made to cover the ground already presented adequately in these and similar reviews.

Illustrative examples of colorimetric tests employed for various vitamins and hormones have been collected in Table VIII. In instances in which extinction coefficient data are not given in the literature, the range of concentrations employed and accuracies claimed by various investigators for their particular experimental conditions are given in the table.

IV. FLUORESCENCE SPECTROSCOPY

1. Methods and Applications

The phenomenon of fluorescence involves the absorption of radiant energy and its re-emission as radiant energy of different wave length. Usually, the excited radiation is of longer wave length (lower frequency) than the exciting radiation (Stoke's law), but this is not universally true. The frequencies of the fluorescent (excited) spectrum bands are characteristic of the fluorescing substance and are independent of the frequency of the exciting radiation, provided the latter is of short enough wave length (high enough frequency) to produce excitation. In this respect, fluorescence differs from the Raman effect, in which the frequency of the scattered radiation deviates from that of the incident radiation by fixed amounts, the deviation, $\Delta\nu$, rather than the scattered-light frequency, $\nu \pm \Delta\nu$, being characteristic of the substance.

In fluorescence spectroscopy, the substance to be examined is excited to fluorescence by incident light of appropriate wave length (the intense line in the mercury vapor arc spectrum near 3650 Å, isolated by an appropriate filter, is frequently used). The fluorescent light is then sent through a spectrograph and photographed or otherwise examined in order to determine the characteristic fluorescent spectrum of the substance in question. The technique and its uses are discussed in detail by Dhéré (96). There has been little application in the vitamin and hormone field. In the isolation of carcinogenic hydrocarbons, however, which are closely related to the steroid vitamins and hormones, fluorescence spectroscopy played a major rôle (97).

In a sense, the mere visual examination of the fluorescence of a substance is an abbreviated fluorescence spectroscopy in which the eye serves as the spectrum analyzer through its ability to distinguish the various colors of visible fluorescent light. The technique, being extremely simple (consisting as it does of the examination of the substance in a darkened room while it is illuminated with ultraviolet light from which visible radiation

TABLE VIII
Colorimetric Tests for Vitamins and Hormones

Substance and Reference	Reagent	Color or Absorption Maximum of Reacton Complex	Quantities Required, and Accuracy
Thiamine (118, 119)	Alkaline diazotate of *p*-amino-acetanilide	Red-purple ppt., soluble in isobutyl alcohol	Min. 5 μg. Acc. ±2%
Riboflavin (120)	Reduction to leuco form by hyposulfite and comparison of absorption of two forms	λ max. 4450 Å. (riboflavin)	Range 2 to 16 μg. per ml. Acc. ±0.6 to ±0.9 μg. per ml.
Pyridoxin (121)	Diazotized sulfanilic acid and *p*-nitroaniline in alkaline solution		Min. 10 μg.
Nicotinic acid (122, 123, 124)	Cyanogen bromide and an aromatic amine (*e.g.* aniline)	λ max. 4000–4400 Å.	Min. 1 to 5 μg.
Ascorbic acid (125, 126, 127)	2,6-Dichlorophenol-indophenol	Green, λ max. ca. 5000 Å.	Range 1 to 15 μg. per ml. Acc. ±1% to ±5%
Vitamin A_1 (69)	$SbCl_3$ in $CHCl_3$	λ max. 6170 Å.	$E^{1\%}_{1cm.}$ = 6000. Computed concentration required = 0.072 μg. per ml. for D = 0.434 Acc. ±0.0007 μg. per ml.
Vitamin A_2 (61, 62)	$SbCl_3$ in $CHCl_3$	λ max. 6930 Å.	
Vitamin A_3 (63)	$SbCl_3$ in $CHCl_3$	λ max. 4960, 5940 Å.	
Vitamins D_2 and D_3 (128, 129)	$SbCl_3$ in $CHCl_3$ or $SbCl_3$ and acetyl chloride in $CHCl_3$	λ max. 5000 Å.	$E^{1\%}_{1cm.}$ = 1800. Min. 0.1 μg. to 2.0 μg. per ml.
Vitamin E (130)	Nitric acid	λ max. 4670 Å.	Min. 0.5 mg. Acc. ±3%
Vitamin K (131)	Sodium diethyldithiocarbamate and alkali in alcoholic solution	"Deep cobalt blue"	Min. 5 μg. per ml.
Oestrone (132)	*m*-Dinitrobenzene and KOH in ethanol	λ max. 5200 Å.	Min. ca. 8 μg.
Oestrone (133)	Diazotized dianisidine	λ max. 4100 Å.	Range 2.5 to 30 μg. Acc. ±10%
Androsterone (132)	*m*-Dinitrobenzene and KOH in ethanol	λ max. 5200 Å.	Min. ca. 8 μg.

has been substantially eliminated by suitable filters) deserves wide application in the qualitative examination of substances. Mercury-arc sources, such as the General Electric BH-4, are available with envelopes of appropriate filter glass which transmit the 3650 Å mercury line readily but absorb most of the visible radiation. Examination of biological fluids by such methods shows the presence of flavins, thiochrome, and various other substances. Qualitative discrimination between such substances is possible from the color of the fluorescence and its behavior toward pH change, solvents, etc., and some degree of quantitative estimation may be made by comparing the intensity of fluorescence with that of a standard substance such as quinine sulfate. Stimson and Reuter (98) have reported data obtained in this way for the fluorescence of various purines and pyrimidines in acid and alkaline solution. Some of these are of interest as possible contaminants in the assay of B_1 by the thiochrome method. Qualitative examination has been used for the estimation of vitamin A in tissue sections, etc., by the method of fluorescence microscopy (99–102).

V. FLUOROPHOTOMETRY

1. *General Considerations*

Fluorophotometry or fluorimetry is concerned in general with the quantitative measurement of the fluorescent light excited in substances under standard conditions, and in particular with the determination of the concentration of substances in solution by the application of such techniques. The relationship between the concentration of a substance in solution and the intensity of the fluorescent light which it emits when excited involves many factors. If two simplifying assumptions are made, however, namely (1) that the absorption of the fluorescent radiation by the substance in solution is negligible and (2) that substantially all of the exciting radiation is re-emitted as fluorescent radiation, a straightforward relationship may be derived for the intensity of fluorescent light as a function of concentration (the intensity of the exciting radiation being constant). To simplify the derivation, absorption coefficients β in terms of $\log_e$, instead of extinction coefficients, K, in terms of $\log_{10}$, will be used. If "I_a" is the intensity lost by absorption in the solution and re-emitted as fluorescent light of intensity "I_f," then (following previous notation)

$$I_a = I_f = I_0 - I_x \qquad (39)$$

But

$$c = \frac{1}{\beta x}(\log_e I_0 - \log_e I_x) \qquad (40)$$

from the Lambert-Beer law. Substituting for "I_x" from (39) in (40):

$$c = \frac{1}{\beta x}[\log_e I_0 - \log_e(I_0 - I_f)] \tag{41}$$

Expanding the second part of the term in brackets in equation (41), namely $-\log_e (I_0 - I_f)$, by Maclaurin's theorem:

$$-\log(I_0 - I_f) = -\log I_0 + \frac{I_f}{I_0} + \frac{I_f^2}{2I_0^2} + \frac{I_f^3}{3I_0^3} + \cdots + \frac{I_f^n}{nI_0^n} + \cdots \tag{42}$$

Substituting this expansion in (41) and simplifying:

$$c = \frac{1}{\beta x}\left[\frac{I_f}{I_0} + \frac{I_f^2}{2I_0^2} + \frac{I_f^3}{3I_0^3} + \cdots + \frac{I_f^n}{nI_0^n} + \cdots\right] \tag{43}$$

If all but the first term of equation (43) could be ignored, "c" would be a linear function of "I_f," thus

$$c = \frac{1}{\beta x}\frac{I_f}{I_0} = AI_f \tag{44}$$

$$\text{where A is a constant } = \frac{1}{\beta x I_0}$$

It is evident from inspection of equation (43) that the terms of higher order than the first are negligible if I_f is small in comparison with I_0, but not if I_f is large. Thus, for $I_f = 0.01\ I_0$, the second term is but 0.5 per cent of the first, and higher order terms are negligible. For $I_f = 0.1I_0$, the second term is still only 5 per cent of the first, but for $I_f = 0.5\ I_0$ the corresponding percentage is 25, and for $I_f = 0.9\ I_0$ it is 45. Hence, in order that the relationship between concentration and fluorescent light intensity be approximately linear it is necessary that the concentration or cell length be small enough so that approximately 90 per cent or more of the incident light passes through the solution unabsorbed (*i.e.* $I_f < 0.1\ I_0$). In the above derivation, it has been assumed for simplicity that all the absorbed light is re-emitted as fluorescence, but essentially the same argument holds if the intensity of the fluorescent light is a constant fraction of the intensity lost by absorption, that is, if $I_f = BI_a$ where B is a constant less than 1. Light loss by reflection has also been neglected, but the introduction of factors for reflection losses and for absorption by the cell does not alter the conclusions. A third factor neglected is the "quenching" of fluorescence by various means—in particular, "self quenching" at high concentration levels. This is of importance with some substances, *e.g.* riboflavin.

The advantages of using small concentrations which result in a nearly linear relationship between concentration and fluorescence are that the calibration of the instrument is simplified and the interpolation between experimental points is made easier and more accurate. If a strictly linear relationship holds and if r_1, and r_2 are the instrument readings for two known concentrations c_1 and c_2, R the reading obtained with a solution of unknown concentration, R_b the reading obtained with a "blank"

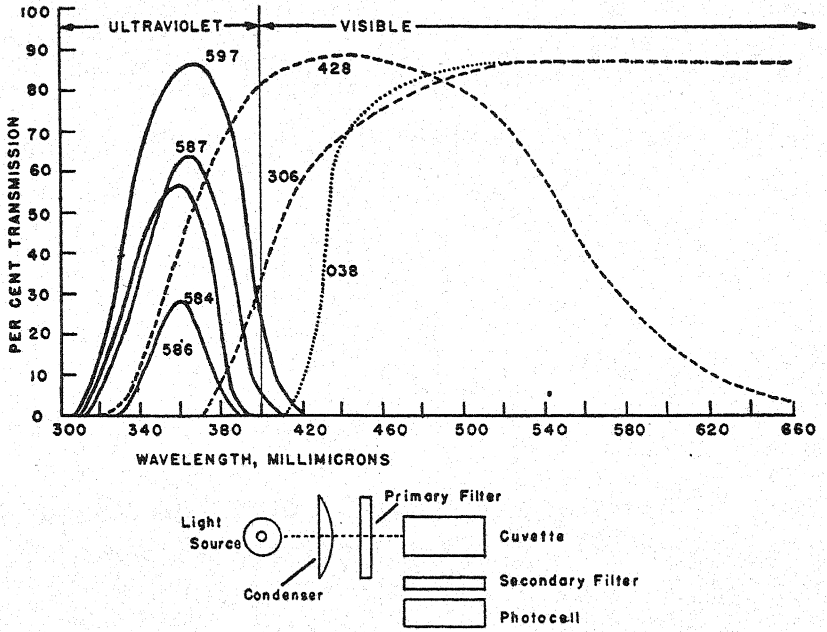

Fig. 14. Transmission curves of filters used in the thiochrome assay of thiamine. Diagrammmatic view of an optical system for fluorophotometry

(cell containing the solvent only), and C the unknown concentration, then

$$C = (R - R_b)\,\frac{c_1 - c_2}{r_1 - r_2} \tag{45}$$

Even if "I_f" is a linear function of "c," non-linearity may, of course, be introduced by the instrument itself.

2. Methods and Precision of Measurement

Visual methods (*e.g.*, modified Duboscq colorimeters) have been employed for fluorophotometry, but photoelectric techniques are almost universally used, and they alone will be considered here. The essential components of a photoelectric fluorophotometer are (1) a light source, (2) a "primary" filter for transmitting the exciting radiation and absorbing radiation in the region corresponding to the fluorescent light emitted, (3) a cell or cuvette for holding the solution, (4) a secondary filter

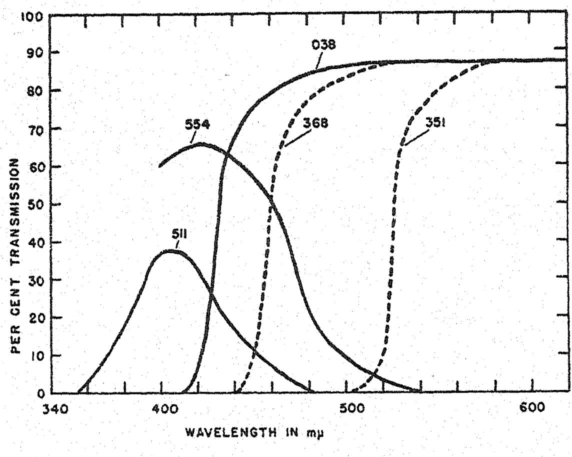

Fig. 15. Transmission curves of filters used in the fluorophotometric assay of riboflavin

TABLE IX

Filters Usually Employed in the Fluorophotometric Assay of Thiamine and Riboflavin

(Numbers refer to filters of the Corning Glass Works)

Data from various references

Substance	Primary Filter	Secondary Filter
Thiamine (thiochrome method)	One of the following: 584 586 587 597	038 followed by 428, or 306 alone
Riboflavin	511, or 511 + 038, or 554	351, or 368

for absorbing scattered exciting radiation and transmitting the fluorescent radiation, (5) a photocell, and (6) an indicating system for determining the response of the photocell. The photocell and secondary filter are customarily placed at right angles to the direction of the exciting radiation beam in order to eliminate insofar as possible the effects of scattered incident radiation (Fig. 14). The light source is usually a high-pressure mercury arc such as the General Electric H-3 or H-4, in a glass envelope, but low-pressure arcs in quartz (*e.g.*, the "Uviarc") are also used. Since wave lengths of 3650 Å or longer, readily transmitted by glass, are ordinarily employed for excitation, the optics may be of glass, but they should be of a kind of glass which does not fluoresce appreciably. The primary and secondary filters most commonly used in the assay of vitamin B_1 and riboflavin are listed in Table IX, and their transmission characteristics are shown in Figs. 14 and 15. Photocells of either the barrier layer or photoemissive type may be employed. The former are connected directly to low resistance galvanometers (the resistance should be less than 1000 ohms for linear response to light with barrier layer cells (103)) of high current sensitivity of the order of 5×10^{-3} microamperes per scale division. The latter may be used to operate microammeters or milliammeters through suitable electronic amplifiers. Details of electrical circuits and instrumentation have been reviewed elsewhere (90, 104, 105).

3. Applications

Fluorophotometry has been used most extensively for the assay of thiamine and riboflavin, and these substances alone will be considered.

Thiamine. When thiamine is oxidized (by $K_3Fe(CN)_6$) an oxidation product, thiochrome, is obtained which has a brilliant blue fluorescence when excited by ultraviolet light (106) (*e.g.*, the 3650 Å mercury arc line). The oxidation product is soluble in isobutanol. This fluorescent conversion product was early made use of in the assay of thiamine (107). Cocarboxylase, which has biological activity equivalent to that of thiamine, yields an oxidation product insoluble in isobutanol. In order that cocarboxylase might be assayed along with thiamine, modifications of the procedure have been introduced (108, 109) whereby cocarboxylase is enzymatically converted to thiamine during extraction of the sample. The thiochrome assay procedure has come into widespread use. Literature regarding recent modifications of the technique, and its application to the assays of foodstuffs and biological fluids will be found in György's review (94).

A study of the precision of measurement obtainable in the thiochrome assay of thiamine with five commercial instruments has been made (105). All the instruments tested were capable of assaying thiamine at levels of 0.007 to 0.1 μg. per ml. of thiochrome solution, with essentially linear response to concentration and with probable errors of the order of 0.0001 to 0.021 μg. of thiamine per ml. of thiochrome solution. A partial summary of the data is given in Table X.

Riboflavin. The yellow-green fluorescence of riboflavin was observed by Kuhn and Wagner-Jauregg (110). The fluorescence is readily excited by blue light in the range of about 4300–4400 Å. If the secondary filter has little transmission below about 5200 Å, scattered exciting radiation (of the above range) is effectively eliminated. The fluorescence is considerably affected by the solvent and by the pH of the solution, being

TABLE X

Tests of Sensitivity and Instrument Error with Five Commercial Fluorophotometers

Data from Loofbourow and Harris (105) and from unpublished results

Nature of Test	How Determined	Instrument				
		A	B	C	D	E
Maximum Sensitivity, μg. per ml. per scale division	With quinine sulphate in 0.1 N H_2SO_4	0.0014	0.0013	0.00092	0.0078	0.00078
	With thiamine as thiochrome	0.00051	0.00047	0.00034	0.0029	0.00029
	With riboflavin	0.00089*				0.0029
andom instrument error, per cent of full scale (20 readings)	Maximum deviation	±1.0	±1.9	±1.7	±2.3	±1.0
	Standard deviation, σ $\sigma = \sqrt{\frac{\Sigma(M-x)^2}{n}}$	±0.68	±1.17	±1.04	±1.09	±0.51
	Probable error (50% chance of occurrence)	±0.46	±0.79	±0.70	±0.74	±0.34
obable error in μg./ml. (50% chance of occurrence)	Thiamine	±0.00023	±0.00037	±0.00024	±0.0021	±0.00010
	Riboflavin	±0.00040*				±0.0010
X Probable error in μg./ml. (0.7% chance of occurrence)	Thiamine	±0.00092	±0.00148	±0.00096	±0.0084	±0.00040
	Riboflavin	±0.0016*				±0.0040

* Computed from data of Conner and Straub (113).

aximum at pH 6 to 7 in the range from 1.7 to 10.2 (111, 112). According Karrer and Fritsche (111) maximum fluorescence occurs at a concentra-on of 0.003 per cent and a pH of 7.0. A linear relationship between ncentration and fluorescence has been found to hold in the range 0.013

to 0.13 μg. per ml. (113). Aqueous solutions are employed for the measurements. The fluorescence can be suppressed reversibly by reduction with sodium hydrosulfite, and restored by shaking the solution with air. This has been made use of in a fluorophotometer technique for assaying riboflavin, in order to eliminate effects of contamination by fluorescent substances which do not reversibly regain fluorescence after reduction (114). Corrections for absorption by pigments may be made by adding known amounts of riboflavin. Details of assay techniques are given in numerous papers (115, 116), of which representative ones only have been cited here (see also György's review (94)).

VI. CONCLUSIONS

A comparison of Tables VI, VIII and X shows, as was indicated in the introduction, that there is a large variation in the applicability of the various techniques discussed to the assay of vitamins and hormones from the standpoint of the sensitivities obtainable. Absorption spectrophotometry is highly sensitive as an assay method for vitamin A and for α- and β-carotene. In general, however, the sensitivity and expected accuracy of the absorption spectrophotometric method are about the same as those for colorimetric tests (Table VIII). The greater simplicity of the latter therefore favors their use in assay procedures. In the case of oestrone and androsterone, colorimetric methods have a clear advantage in sensitivity and accuracy (about 10:1) over absorption spectrophotometry. In the case of thiamine and riboflavin, fluorophotometry is by far the most sensitive of the assay methods. Thus about 1000 times as much thiamine and 100 times as much riboflavin are required for accurate assay by spectrophotometry as by fluorophotometry.

The advantage of absorption spectrophotometry in following isolation procedures, in the identification and characterization of the substances in question, and in determinations of purity is, on the other hand, quite evident. The complexity of the spectra of the various substances, as indicated in the figures, and the effects of such influences as solvents and pH on the spectra result in detailed data especially useful in characterization and identification.

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* Due to delays arising from the war, it has not been possible to examine a copy of the new edition of Morton's book on the absorption spectra of vitamins and hormones (117) in the preparation of this review. The earlier edition (53) has been a standard reference for some years past, and the revised edition should be a useful source of information in this field.

The Chemical and Physiological Relationship between Vitamins and Amino Acids

By H. H. MITCHELL

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I. INTRODUCTION

The importance of amino acids as the building stones of the proteins of which protoplasm is so largely composed is generally realized. It is perhaps not so generally realized that their functions in animal metabolism extend far beyond this structural function. Some of the tissue proteins, either by conjugation with an active prosthetic group, or by some slight modification in structure, assume the rôle of enzymes (1) and catalyze many digestive and metabolic reactions. Many proteins, protein derivatives, and amino acid derivatives are to be found among the hormones, that important class of tissue products concerned with the regulation of metabolism and the proper use of food. In immunity, resistance to disease, and chemical defense against invasion of the body by alien substances, specific proteins are elaborated possessing very specific functions. These antibodies appear to be merely modifications of serum globulin (2). Amino-acid derivatives, such as creatine, carnosine, glutathione, and ergothioneine, serve many important functions in the various tissues of the body, while the bile acids, originating in part from cystine and glycine, perform important duties in the digestion of fats and the absorption of many nutrients soluble in fat solvents.

This is not a complete enumeration of the functions of amino acids, amino-acid derivatives and amino-acid aggregates in the animal body, but it is sufficient to indicate their omnipresence and their intimate association with most of the physiological activities characteristic of life. It is, therefore, not surprising that no one of the vitamins is peculiarly associated with amino-acid or protein metabolism, such as the association of thiamin with carbohydrate metabolism, of choline with fat metabolism, and of vitamin D with the metabolism of calcium and phosphorus. Rather, it may be expected that for each vitamin there are many points of contact with amino acids and proteins and many interrelationships. The surprising feature of this situation is that so few of these contacts and interrelationships have been thus far elucidated by physiological and biochemical research. Possibly their very number renders their detection difficult, since it seems unlikely that a deficiency of any one vitamin or group of vitamins will induce such a serious breakdown in amino-acid metabolism as to occasion a dramatic disturbance of health, a deficiency disease, curable or preventable by some simple dietary alteration, the nature of which will reveal a vital amino acid-vitamin relationship. It will be the purpose of this chapter to discuss the somewhat fragmentary and often inconclusive evidence that has been revealed concerning the existence and the nature of chemical and physiological relationships between the various vitamins on the one hand, and the amino acids and their functional derivatives and combinations on the other.

II. Chemical Relationships between Vitamins and Amino Acids

1. *General Considerations*

From his early work (3) indicating that the substance in rice polishings that is curative of polyneuritis in birds is an organic base, Funk (4), somewhat rashly, concluded that all of the deficiency diseases, with the exception of pellagra, can be prevented and cured by the addition to the diet of certain substances of the nature of organic bases, precipitable by phosphotungstic acid and similar precipitants, for which he proposed the name "vitamines." We now know that a number of the vitamins, although not all of them by any means, contain nitrogen with basic properties, including thiamin, containing the pyrimidine and thiazole rings, nicotinic acid and pyridoxin, containing the pyridine ring, riboflavin, containing the alloxazine ring, pantothenic acid, containing β-alanine, biotin, containing a cyclic urea structure, together with choline and *p*-aminobenzoic acid. Conceivably these vitamins may have originated in part from amino acids. However, any extended consideration of the relationship of amino acids to the biogenesis of vitamins can hardly be attempted here. As the vitamins relate to animal nutrition, a consideration of their synthesis would be paradoxical, since vitamins may be defined as essential nutrients that must be contained in the diet, either as such or as some closely related precursor. Their biogenesis thus becomes a problem in plant nutrition, a subject beyond the competence of the reviewer to discuss critically. The synthesis of vitamins in the alimentary canal of the animal by symbiotic microörganisms, particularly in the rumen of the ruminant and in the cecum of other animals, would logically come within the limits of this chapter in so far as the synthetic reactions involve amino acids, but unfortunately, beyond indicating definitely that vitamin synthesis occurs where food residues stagnate and ferment, investigation has not disclosed the nature of the reactions involved.

These general considerations, however, do not entirely remove from proper consideration here all questions involved in the biogenesis of the vitamins.

2. The Relation of Amino Acids to the Biogenesis of the Vitamins

The thiamin molecule consists of two parts, containing the pyrimidine ring and the thiazole ring. From the fact that the synthesis of the vitamin from its two component parts may be accomplished *in vitro* and *in vivo* (5, 6), it may be presumed that this process represents the last step in the biogenesis of thiamin. The presence of the $—CH_2—CH_2 \cdot OH$ side chain in the thiazole component of the molecule suggests the possible biological formation of this compound from the corresponding α-aminopropionic acid, in a manner similar to the formation of β-phenylethyl alcohol from phenylalanine, of tyrosol from tyrosine, and of tryptophol from tryptophan in yeast fermentation. The corresponding amino acid, β-(4-methylthiazolyl-5)-alanine, has been synthesized (7, 8), and has been shown to be convertible to thiazole by the pea root (8) and by yeast (9).

In this discussion, and throughout this volume, choline occupies a peculiar position. It may be synthesized by the animal from other dietary components, and hence may be considered not to be a vitamin. On the other hand, under certain conditions the body cannot supply the demand for choline by the tissues and it must be supplied preformed in the diet. To this extent it may be considered a vitamin, and possibly also because a considerable fraction of its molecule cannot be synthesized by animal tissues. Since choline is synthesized from intermediate compounds in the tissues of the animal, it may be considered to be a hormone. For the purposes of this chapter, we will consider choline to be a vitamin.

The precursors from which the rat synthesizes choline have now been identified. Du Vigneaud and others (10) have shown that the methyl groups are derived from methionine, while Stetten (11) has proven that ethanolamine is the compound so methylated. The rat can manufacture ethanolamine by the reduction of glycine (11), or possibly by the decarboxylation of serine (12). Using isotopically labeled ethanolamine, in diets rich and poor in labile methyl groups, Stetten (13), by comparison of the ratios of isotopic nitrogen in the ethanolamine and choline isolated from the phosphatides of the experimental rats, showed that the conversion of ethanolamine to choline proceeds without hindrance even when the diet is sufficiently poor in labile methyl groups to cause fatty livers. This finding explains the independence of the choline content of the body and that of the diet.

The rat can synthesize nicotinic acid (14, and others), and hence for this animal nicotinic acid is not a vitamin. The manner of its synthesis in the rat has been studied by Huff and Perlzweig (15), based on a hypothesis proposed by Guggenheim (16), which pictures the synthesis as follows:

$$\begin{array}{c}\text{Ornithine}\\ \downarrow\uparrow\\ \text{Proline}\end{array} \underset{+H_2}{\overset{-NH_3}{\rightleftharpoons}} \delta\text{-Aminovaleric acid} \xrightarrow{-H_2}$$

$$\delta\text{-Aminodehydrovaleric acid} \xrightarrow[-H_2O]{+CH_2O} \text{Guvacin} \xrightarrow{-H_2} \text{Nicotinic acid.}$$

The excretion of nicotinic acid and trigonelline was determined in rats on a nitrogen-free diet, and on the same diet plus choline, a donor of methyl, *dl*-δ-amino-*n*-valeric acid, or glycine, for conjugation with nicotinic acid to form nicotinuric acid. With these dietary supplements, the excretion of nicotinic acid derivatives in the urine promptly increased, followed by a decrease, not so prompt in all cases, on their removal from the diet. A casein supplement led to an increased excretion of trigonelline in male but not in female rats. The experiments do not afford any convincing evidence that the Guggenheim hypothesis represents the main (or only) pathway for nicotinic acid synthesis.

III. GENERAL PHYSIOLOGICAL RELATIONSHIPS BETWEEN VITAMINS AND PROTEINS

1. As Indicated by Appetite Studies

Interesting relationships between vitamin intake and the metabolism of amino acids are suggested by the studies of Richter and associates on the effects of various vitamin deficiencies on the appetite of rats for the organic nutrients, proteins, carbohydrates, and fats. Rats in an acute stage of B complex deficiency (polyneuritis, facial dermatosis, diestrous vaginal smears) showed a marked aversion for carbohydrates and protein, and a craving for fat (17). When yeast was given as a curative for the multiple avitaminotic condition, the appetite of the rats returned to normal.

In later experiments (18, 19), the effects of various components of the vitamin B complex on appetite were studied. On single additions of these components to the diet, or on combinations of them, the protein appetite varied widely. On riboflavin, nicotinic acid, or pyridoxin alone, or on thiamin and pyridoxin, or on riboflavin and pyridoxin, the protein appetite was minimal. A combination of thiamin and riboflavin had a definitely favorable effect upon the protein appetite, which was increased further by successive additions of pyridoxin and nicotinic acid. However, a combination of all four of these factors did not raise the protein appetite, even approximately, to the level attained following a yeast supplement. The experiments are interpreted as indicating a marked effect of thiamin, and particularly of riboflavin, on protein (casein) appetite, but also a marked effect of other yeast components than those studied individually.

Elsom and Machella (20) observed that normal human subjects, receiving an adequate self-selected diet, exhibited a marked positive correlation between their intakes of thiamin and of protein, a correlation that did not seem to be merely an expression of the fact that foods rich in protein are, by and large, also rich in thiamin.

The aversion of thiamin-deficient rats to carbohydrate may be harmonized with the profound disturbance in carbohydrate metabolism in thiamin deficiency. The aversion to protein may be viewed in a similar way, since so many of the proteinogenous amino acids are convertible into glucose. But the favorable effect of riboflavin on protein appetite is not so easily reconciled with available information on the metabolic functions of riboflavin.

2. In Digestion

The effect of various avitaminotic conditions on the digestibility of protein and other organic nutrients has been studied by St. Julian and Heller (21), using rats and guinea pigs that had been depleted in vitamins

A, B_1, B_2, C or D. The coefficients of apparent digestibility in such prepared animals were compared with those obtained with normal animals on the same rations except for the appropriate vitamin additions. In the case of each of these vitamins, the coefficients of apparent digestibility of protein, as well as of fat and carbohydrate, were unaffected by its presence in, or absence from, the diet. Braman, *et al.* (22), in experiments having a much broader significance, also failed to detect any significant effect of chronic partial deficiencies of vitamins A, D and G on the digestibility of protein. McClure and others (23), in experiments apparently involving partial deficiency of vitamin B_1 in rats, observed a slight, but statistically significant, increase in the digestibility of protein in the deficient rats compared with their normal controls.

Cowgill and Gilman (24) obtained some evidence with dogs that a dietary deficiency in vitamin B_1 may impair the secretion of gastric juice, but none that vitamin B_2 was effective. The negative results with vitamin B_1 reported by Alvarez and others (25) on human subjects may be explainable on the basis that the subjects were not sufficiently depleted in the vitamin to constitute a conclusive test, as evidenced by the continued good appetite throughout the six weeks of the experiment.

Observations on the protease concentrations in the digestive glands of polyneuritic pigeons by Farmer and Redenbaugh (26) indicated an impairment of 40 per cent as compared with normal controls. Sure and associates (27) were quite unable to confirm these results in experiments on rats, except after a succession of vitamin B_1 depletion periods, when a reduction in peptic activity was noted. However, the conclusion was not supported by an adequate statistical analysis of the data. Negative results were obtained with vitamins A and G. Negative results have also been reported (28) on the effect of avitaminosis C on the activity of pepsin. Colloidal solutions of carotene have been observed (29) to inhibit the activity of pepsin and trypsin.

Relationships of vitamins and amino acids in digestion include also the effect of bile acids on the absorption of the fat-soluble vitamins. The main bile acids are glycocholic acid, a combination of glycine and cholic acid, and taurocholic acid, a combination of taurine and cholic acid. Taurine is probably derived in the body from either cystine (30) or methionine (30, 31). The intestinal absorption of vitamin D (32, 33) and vitamin K (34) is definitely dependent on the presence of bile or bile salts. There is evidence that the absorption of vitamin E and of *p*-aminobenzoic acid is also dependent upon bile acids, though in the case of vitamin E desoxycholic acid, rather than taurocholic or glycocholic acids, were used (35). Desoxycholic acid plays an essential rôle in the absorption of carotene (36), but the evidence on the efficacy of sodium glycocholate is divided (37, 38). The absorption of vitamin A may occur in the absence of bile (39), but may possibly be accelerated by the presence of bile salts (40). To what extent these relationships between bile salts and vitamin absorption from the intestinal tract involve combinations between vitamin and bile acids is not known, but at least for vitamin K, surface tension activity is important (41).

3. *In Metabolism*

The effects of vitamin deficiencies upon the metabolism of nitrogen should afford some evidence of the existence of general relationships between vitamins and amino acids in animal physiology. An immense amount of work of this general nature has been reported in the literature, but much of it is of dubious significance at the present time, because of improper control of experimental conditions, particularly with reference to the intake of food. This particular defect inevitably confuses inanition effects with *bona fide* effects of avitaminosis. Much of the earlier work unavoidably presents difficulties of interpretation for the reason that the effects observed cannot be traced to specific vitamins in the present-day category. Therefore, the following discussion will be restricted to the better controlled experimental reports and largely to the more recent work possessing the greater significance.

Working in Mendel's laboratory, Karr (42) in 1920 reported an interesting experiment in which a dog subsisted on a constant amount of a diet entirely devoid of water-soluble vitamins for 60 days with little loss in weight and with no impairment in the digestion of nitrogen or in the retention of this element. On the 60th day symptoms of polyneuritis developed. Sherman and Gloy (43) found that the survival time of rats on diets very deficient in all water-soluble vitamins was not appreciably modified by variations in dietary protein ranging from 12 to 54 per cent. However, in a later paper from the same laboratory (44), it was observed that experimental rats receiving diets of liberal protein content were less severely affected by shortage of vitamin B_2 than were animals on a lower plane of protein nutrition. On the basis of these results, the authors were inclined to revive the theory of Goldberger and Tanner that the protein supply, either quantitatively or qualitatively, is involved in the etiology of pellagra. This misinterpretation of their experiment on rats is quite excusable in the light of later developments in pellagra research, although the program of *ad libitum* feeding followed seems a sufficient barrier to any precise interpretation whatever. In carefully controlled experiments on rats apparently involving a study of what we now call vitamin B_1, McClure, Voris, and Forbes (23) observed no significant effect of a partial deficiency of the vitamin on the retention of dietary nitrogen by growing rats. Dogs on a diet more severely deficient in vitamin B_1 were observed by Lewinson (45) ultimately to go into negative nitrogen balance, though sometimes not until after 100 days, even though the intake of food was maintained constant. Lavrov and Yarusova (46) obtained somewhat similar results upon polyneuritic pigeons. Sure and Kik (47) found no significant alteration in the non-protein nitrogen of the blood of rats on B-complex deficient, or B_1 deficient, diets when the intake of food of control and test animals is properly controlled, even though the pathological animals lost as much as 30 per cent of their body weight.

In paired feeding experiments with rats or chicks, Kon (48), Braman, and others (22), Sure (49), and Kleiber and Jukes (50) prove very conclusively that a deficiency of vitamin B_2 (riboflavin) impairs growth and restricts the extent to which dietary nitrogen is used in growth. The latter experiment on chicks showed that for each gain to the body of 100 calories of energy, the flavin-deficient chicks gained 2.3 ± 0.7 g. of nitrogen, while their flavin-supplied controls on equal food intake gained 5.1 ± 1.4 g. of nitrogen. Braman and coworkers (22) also showed similar effects of a deficiency of vitamin A, but not of vitamin D. The observations of Basu and Gupta (51) to the effect that the biological value of autoclaved casein is definitely increased in mature rats by dietary deficiencies of either vitamin A, B_1, B_2, B complex or D are quite incomprehensible and can hardly be accorded credence until confirmed in another laboratory. Yarusova (52) and Shipp and Zilva (53) report no indications of a disturbed absorption or retention of nitrogen in guinea pigs during the early stages of the development of scurvy.

The experimental results discussed in the preceding paragraph indicate some connection, if only when pathological conditions develop, but particularly with reference to riboflavin, between vitamins and the most efficient utilization of amino acids, but whether the connection is direct or indirect is a problem that remains unsolved. The situation is well expressed by Kon (48) in evaluating his own data: "In conclusion it may be said that while vitamin B_2 is undoubtedly in some way linked with the metabolic processes of the body, it is not at all evident whether this connection is to any extent of a different type from that of the general dependence of normal metabolic exchange upon the availability of any one of the indispensable food ingredients." This remark applies with particular force to the experiments of McHenry and Gavin (54) on the relationship of pyridoxin to the synthesis of fat from protein. Using various components of the B complex as supplements to a basal diet high in protein and devoid of fat, carbohydrates and the B vitamins, and feeding their rats *ad libitum*, these authors observed that only when pyridoxin was among the dietary supplements were appreciable amounts of fat stored in the carcasses above those of the controls, but also only in these cases did any growth occur. The predominant deficiency of the basal diet with reference to growth was evidently a deficiency in pyridoxin. With this deficiency supplied by dietary supplementation, increased growth occurred with an increased deposition of fat, since fat deposition is a concomitant of growth. Therefore, the experiments cannot be said to demonstrate a direct or intimate relationship between pyridoxin and the conversion of protein to fat.

The indications above revealed that the connection between riboflavin and protein metabolism may be closer than that of any of the other vitamins studied is strengthened by the observation of Kensler and others (54a) that riboflavin and casein alone exerted a protective effect in rats against liver carcinogenesis induced by dimethyl aminoazobenzene (butter yellow) of only 20 to 30 per cent, but when both were added simultaneously to the basal diet, the resulting protection amounted to 97 per cent. Of the same significance are the findings of Sarett and coworkers, reported in abstract only (55), that the urinary excretion of riboflavin by growing dogs during periods of low-protein intake (1 g. per kg. body weight) was about five times that found during periods of moderate- and high-protein intake (7 g. and 12 g. per kg.), and also that of equal intravenously injected doses of riboflavin, 70 per cent was excreted during periods of low protein intake, 27 per cent during periods of medium protein intake, and only 3 per cent during periods of high protein intake. Less pronounced and consistent correlation was noted between the plane of protein nutrition and the excretion of nicotinic acid and pyridoxin, and none at all with the excretion of thiamin.

IV. SPECIFIC PHYSIOLOGICAL RELATIONSHIPS BETWEEN VITAMINS AND PROTEINS

The association of many vitamins with tissue proteins is evident when attempts are made to extract them from tissues as an initial step in their chemical or microbiological determination. The total vitamin content can be removed only after procedures that involve the cleavage of protein linkages, such as autolysis, acid hydrolysis, or enzymic proteolysis. This is known to be true of thiamin, riboflavin, biotin (56, 57), and pyridoxin and quite probably is true of pantothenic acid (58) and ascorbic acid (60, 61). It has recently been shown (62, 63) that approximately half of the thiamin in milk is bound more or less firmly with protein. Choline, combined in the phosphatides lecithin and sphingomyelin, and ethanolamine, a probable derivative of choline, combined in cephalin, are associated with protein molecules in the lecithoproteins (64). The thromboplastic protein from the lung, involved in the "activation" of prothrombin in the blood, is a conjugated protein of this description (59, 65). The probable combination of serine with ethanolamine in the cephalin molecule is another point of contact between a vitamin (choline) and an amino acid (66, 67).

The nature and functions of some of these protein-vitamin combinations are known or suspected with some degree of probability.

1. The Flavoproteins

It is not within the province of this chapter to discuss the physiological rôle of the flavoproteins. The rôle of these enzymes in biological oxidations has been recently reviewed by Ball (68). At present some ten catalytically active flavoproteins are known, including two recent additions, liver aldehyde oxidase (69) and cytochrome reductase (70, 71). More recently, Axelrod, Potter, and Elvehjem (72) have brought forth presumptive evidence that succinic dehydrogenase is also a flavoprotein. Having previously shown that the concentration in liver and kidney of *d*-amino acid oxidase (73) and in liver of xanthine oxidase (74), two known flavoproteins, is lowered in riboflavin deficient rats, they report that an inadequate intake of riboflavin definitely depresses the concentration of succinoxidase of the liver, but not of the kidney cortex, brain, heart, or muscle. The mere addition of riboflavin to the incubating mixture does not modify the catalysis of the oxidation of *d*-alanine by *d*-amino acid oxidase (75), indicating that the vitamin acts only in combination with protein.

The discovery of the first flavoprotein by Warburg and Christian in 1932, the so-called "yellow enzyme," led to the general recognition of the close relationship existing between vitamins and oxidative enzymes. The flavoproteins are conjugated proteins, with a prosthetic group consisting of a dinucleotide that may be looked upon as a combination of riboflavin phosphate and adenylic acid, except in the case of the original yellow enzyme, whose prosthetic group is a mononucleotide, riboflavin phosphate. The linkage between the flavin nucleotides and the protein is probably through the isoalloxazine ring and is a fairly stable one.

Flavoproteins play an important rôle as mediators in oxidations initiated by the pyridine nucleotides.

2. Carotenoid Proteins

Carotenoid proteins have been known for many years (76) and many new ones have been identified in recent years. Because of their physiological importance, attention will be restricted to those carotenoid proteins having photosensitive properties and located in the retina of the eye. An extended discussion of the chemistry of the visual substances has been published by Hecht (77). The brief description here given is largely taken from this report.

Rhodopsin is a conjugated protein with a carotenoid prosthetic group called retinene. It is estimated that one molecule of rhodopsin, or visual purple, contains 10 prosthetic groups and that each such group contains one molecule of vitamin A. Rhodopsin is not soluble in the ordinary protein solvents, being similar in this respect to the neurokeratins in the retinal rods. It is found in the rods of the retina of all vertebrate animals except certain fishes.

On exposure to light, rhodopsin dissociates into protein and retinene (visual yellow) and finally to a colorless mixture of protein and vitamin A. In the dark, regeneration of rhodopsin occurs. In the living animal, this regeneration is going on constantly, but depends for its continuance on material, including vitamin A, provided by the circulation. This visual cycle, therefore, is an expender of vitamin A. Being confined to the rods, visual purple is not essential to clear vision in bright light, but rather to vision in dim light. However, since vitamin A deficiency influences the cone threshold to light, as well as the rod threshold, it may be inferred that there is a visual pigment in the cones containing a carotenoid prosthetic group.

In fishes that can live for some time in fresh water, the visual substance differs from rhodopsin chemically, particularly in the substitution of vitamin A_2 for vitamin A in its prosthetic group. It has been called porphyropsin (78). The sea lamprey also possesses this visual pigment (79). The visual cycle of porphyropsin is quite analogous to that of rhodopsin.

3. The Avidin-Biotin Relationship

There exists in egg white a basic protein that combines so firmly with biotin as to render it unavailable to yeast and in the natural digestive processes of the animal. The isolation and properties of the protein have been described by Woolley and Longsworth (80), and its crystallization and analysis have been effected by Pennington, Snell, and Eakin (81). It has been found to be a glycoprotein, similar in biological activity to the concentrates previously prepared from egg white by Eakin, Snell, and Williams (82, 83) and called "avidin." The combination of avidin and biotin is disrupted by heat (steam sterilization). This inactivation of biotin by the protein avidin explains fully the etiology of "egg white injury" in chickens, rats, and man (84) induced by the consumption of considerable amounts of unheated or insufficiently heated egg white. The fact that the pig (85) is able to thrive on a ration containing 4.9 pounds daily of eggs that had failed to hatch after 21 days of incubation may indicate either that this animal does not need biotin, or that its digestive enzymes are able to break up the avidin-biotin combination, or that this combination is destroyed during incubation, even though embryonic development does not occur. The digestibility of the protein of raw egg was just as complete in the pig as that of cooked egg, and no diarrhea resulted from this unusual ration in contrast to experiences of Bateman with the dog.

The biotin in egg yolk is present in a high-molecular, undialyzable form that is physiologically active in yeast growth, but the avidin in egg white is present in such amount as to be able to bind twice as much biotin as the yolk contains (86). It is anomalous that the proteases of the gastro-intestinal tract of the animal are unable to break up the avidin-biotin combination, whereas when introduced parenterally this compound is broken up readily and is actually of therapeutic value because of the biotin thus liberated (87). The compound is not available to bacteria, and a correlation has been found between the biotin requirement of a microörganism and its growth inhibition by avidin (88). Since biotin is present in malignant cells in much greater concentration than in normal cells, it has been suggested (89) that induced biotin deficiency may explain the occasional observance of spontaneous recessions in malignancy, and that raw egg, with its content of avidin, may be a new therapeutic agent, because of its ability to deprive malignant cells (and pathogenic bacteria also) of a life-essential factor. Other proteins than avidin have been found to possess a slight biotin-binding capacity (80).

It has been suggested by Woolley (90) that avidin is but the first to be isolated of a class of nutritionally important substances that might be called anti-vitamins, whose function is to act as governors for the biological processes participated in by the vitamins, and that another instance of vitamin binding is the cause of the deficiency disease in foxes produced by the consumption of raw fish and first described by Green and associates (91). Pathologic findings, limited therapeutic field trials with thiamin and histories of ranch outbreaks confirm the experimental evidence that the disease, Chastek paralysis, is fundamentally a thiamin avitaminosis. The work of Green, *et al.* on foxes was confirmed with chicks (92) and it was further shown that inactivation of thiamin occurred within the feed mixture itself. A water or sodium chloride extract of carp slowly destroys thiamin, as indicated by a yeast growth test (90); the destruction must therefore proceed further than the splitting of the molecule into the pyrimidine and thiazole halves, since in yeast growth the halves are as active as thiamin itself. Whether the action is an enzymic degradation or a slow formation of a thiamin-antithiamin complex has not yet been established.

4. Ascorbic Acid and Collagen Formation

Wolbach (93) has defined the function of ascorbic acid, from the standpoint of the pathologist, as the production and maintenance of intercellular substances: the collagen of all fibrous tissue structures, the matrices of bone, dentine, and cartilage, and probably all non-epithelial cement substance, including that of the vascular endothelium. The relationship of ascorbic acid to elastic tissue and elastin has apparently not been studied. King (94) in his recent review assigns two specific rôles that appear to have been clearly established to vitamin C in animal tissues: a respiratory function, the significance of which in metabolism is still obscure, and "regulation of the colloidal condition of intercellular substances as shown by Wolbach and associates."

Wolbach and Howe (95) showed that scurvy is due to the inability of the supporting tissues to produce and maintain intercellular substance, presumably because of the absence "of an agent common to all supporting tissues which is responsible for the setting or jelling of a liquid product," and that following the administration of anti-scorbutics easily demonstrable amounts of intercellular substance are formed within 24 hours. Later, Menkin, Wolbach, and Menkin (96) proved that ascorbic acid was the effective reparative agent. Following its administration orally or parenterally to scorbutic guinea pigs, there is a renewal of dentine formation in the incisor teeth and a deposition of osteoid matrix and chondromucin at the costochondral junction.

The importance of ascorbic acid in bone development and maintenance (97, 98) in dentine formation in the tooth (99), but not in enamel formation (100), and in the healing of wounds (101) has been observed and reported and generally ascribed to its intimate association with the processes involved in the formation of intercellular substance and collagen. But the evidence, or its interpretation, is not all in conformance with this theory. Hass and McDonald (102), in a study of the deposition, maintenance, and resorption of collagen *in vitro*, using tissues from healthy guinea pigs and rats, found no correlation between the ascorbic acid content of the plasma medium and the production of collagen in their isolated systems, although the content ranged from negligible to above normal values. However, the data were not interpreted as being contrary to the Wolbach theory, as they should not necessarily be, but rather as indicating clearly "that the state of tissues and fluids *in vitro* was not comparable in one or more respects with the state of similar materials in the disease known as absolute scorbutus."

Ham and Elliott (97), however, in their study of bone and cartilage lesions of protracted moderate scurvy, interpret their own data contrary to the jellation theory of Wolbach and Howe and devote considerable space to a critical consideration of the evidence brought in support of it. It is beyond the competence of this reviewer to discuss this controversy intelligently. Suffice it to say that Ham and Elliott do not deny the fundamental rôle of ascorbic acid in collagen deposition; the disagreement with

Wolbach and Howe rests in the manner in which this function is mediated. Ham and Elliott refuse to commit themselves to any particular modus operandi, but prefer to cloak their ideas in the quite invulnerable statement that "the lesions of bone and cartilage can be explained by a theory that postulates vitamin C to be somehow concerned in the metabolic processes of cells."

Whether the progressive accumulation of fibrous tissue and collagen in the muscles in nutritional muscular dystrophy of rabbits (103) is an indication of the implication of ascorbic acid in this disease, is an interesting possibility. The effect of ascorbic acid and hydrogen peroxide in causing an irreversible reduction of viscosity of solutions of collagen and thymonucleohistone (104) and promoting the degradation of synovial fluid mucin (105) suggests the participation of this vitamin in protein degradation as well as protein deposition.

5. *Enzyme-Coenzyme Relationships*

The purpose of this and the following sections is not to present even a bird's-eye view of the relationships between enzymes and vitamins. Such a procedure would encroach upon the subject matter of other chapters in this volume. The intention is merely to point out that these relationships exemplify still further the many points of contact in tissue structure and tissue function between vitamins and amino acid aggregates, the proteins. For enzymes, in so far as their structure has been established by the biochemist, are proteins. They may be produced by living cells from inert proteins, possibly by rather slight changes induced by change of pH, by simple hydrolysis, by catalysis with other enzymes, or by autocatalysis (1). They function as catalysts in specific chemical reactions. The coenzymes, like the enzymes, are catalysts, but they are of lower molecular weight than proteins and unlike the proteins they are heat stable and dialyzable. A number of them have been shown to be derivable from vitamins, which constitute the active group in the molecule (106). They all seem to contain phosphoric acid and to fall within the class of compounds called nucleotides, characteristic of nucleoproteins and the cell nucleus. As Baumann and Stare (106) say: "Cytologists have long maintained that the nucleus is the controlling, physiologically dominant part of the cell. Geneticists have placed the dominance of the nuclear material on the chromosomes and their genes. The demonstration of the rôle of certain coenzymes, nucleotides, in biological oxido-reductions is an interesting biochemical counterpart of the cytological interpretation of nuclear function."

In diphosphopyridine nucleotide (coenzyme 1) and triphosphopyridine nucleotide (coenzyme 2), the enzymatically active group is nicotinic acid amide, the anti-pellagra vitamin. They are both involved in the transfer of hydrogen from several types of substrates to enzyme dehydrogenases which in turn implement the union of hydrogen with oxygen, probably with the intervention of metallo-protein complexes capable of the appropriate valence change. The substrates thus oxidized may include amino acids (glutamate) or amino acid catabolites (lactate). In addition, the diphosphopyridine nucleotide may function in the transfer of the phosphate radicle in various phosphorylation reactions.

In cocarboxylase, the active group is thiamin. It contains one molecule of thiamin and 2 molecules of phosphoric acid combined as the pyrophosphate. It functions with the enzyme carboxylase in decarboxylating pyruvic acid, a metabolite of alanine, of cystine (107), and possibly other amino acids. It also functions as coenzyme in the tissue oxidation of α-ketoglutaric acid (108), a metabolite of glutamic acid. The oxidation and utilization of this substrate is retarded in the tissues of avitaminotic rats and is increased by thiamin addition.

The active group of the coenzyme of *d*-amino acid oxidase is alloxazine-adenine dinucleotide and contains the vitamin riboflavin. Together the protein and the coenzyme, of which the oxidase is composed, catalyze the oxidative deamination of the enantiomorphs of many of the naturally occurring amino acids.

6. *Vitamin-Enzyme Relationships*

Other relationships between vitamins and enzymes have been observed than the coenzyme-enzyme relationship. One example is the oxidation of ascorbic acid by a copper-protein complex, the "ascorbic acid oxidase" (109), the action of which, however, is not confined to this reaction. Ascorbic acid, in the presence of a small amount of copper, augments the activity of the enzyme arginase (110), the protein nature of which has been rendered probable recently (111), though the interaction may be more complicated than this simple statement implies (112). To this extent, ascorbic acid may modify what seems to be the main urea-producing mechanism of the body. Carotene has been observed to exert an opposite effect on this enzyme (113). *p*-Aminobenzoic acid retards the oxidation of tyrosine by tyrosinase, but accelerates the oxidation of *p*-cresol (114). The well-known effect of vitamin D in lowering the serum phosphatase concentrations in rachitic animals (115) is another illustration of the relationships under discussion. Ascorbic acid seems to be more generally involved in these relationships than the other vitamins, as evidenced by reports of its activation of serum phosphatase (116, 117), cathepsin (118), and papain, working in combination with iron (119, 120). The work of Harrer and King (121) on enzyme activity in the tissues of scorbutic guinea pigs, showed a progressive decrease in liver esterase activity during the development of the deficiency, and a marked drop in succinic dehydrogenase activity of heart and skeletal muscle. It is interesting to compare the latter effect with analogous events in riboflavin deficiency (72), in which the succinic dehydrogenase activity of the liver, but not of the heart and skeletal muscle, was depressed. In such experiments as these, in which dietary conditions are imposed that ultimately impair the appetite of the experimental animals, it would seem wise to test the effect of dietary restriction alone (undernutrition) on enzyme concentration, since it has been shown that such an effect may eventuate (72). King (122) is inclined to the view that ascorbic acid in animal tissues acts "as a regulating and protective agent, perhaps exerting an effect upon other more effective hydrogen carriers and other important enzyme systems, rather than functioning as a major carrier itself."

7. Vitamins and Immunity

In so far as immunologic reactions involve the formation of proteins and in so far as these reactions are influenced by vitamins, such reactions fall within the limits of this chapter. But no comprehensive treatment of the subject can be attempted, both because the available information within the restrictions so defined is disconnected and much of it controversial, and because it would be presumptuous for this reviewer to stray for long so far from his own limited field of study.

Vitamin C seems to be related to the complement content of the blood, as revealed by a correlation up to a certain level of ascorbic acid in the blood serum of guniea pigs, between the ascorbic acid content and the complementing activity (123), the relationship holding both *in vivo* and *in vitro.* The same investigators report (124) that ascorbic acid activates reconstructed complement and effectively reactivates aerated complement. The vitamin also exerts a stimulating effect on anti-body production (125), much more so than does nicotinic acid (126).

Ascorbic acid has been claimed to neutralize diphtheria toxin (127 and others), but confirmation has not been always (and lately) successful (128, 129, 130). King and coworkers have reported some protective effect of large doses of ascorbic acid against tooth injury induced in guinea pigs by injection of diphtheria toxin (131). Perla and Marmorston (132) have critically reviewed the literature on the rôle of vitamin C in resistance to disease with the general conclusion that much of it is indecisive and not readily interpretable because the conditions of the experiments, inevitably or otherwise, are too involved and complicated. Perla (133) has also reviewed the rôle of the B vitamins in resistance to disease, finding more indications of positive effects, though such indicated effects seem to be indirect. Feller and others (134) have reported the results of studies on human subjects of the influence of vitamins A and C on certain immunological reactions, using diets deficient in one or the other of these vitamins for periods ranging from 6 to 26 weeks. In the words of the authors: "Certain variations in the results of the immunological tests occurred but these alterations were either no more marked than those occurring in normal persons or were adequately explained on some basis other than a deficiency or sufficiency of vitamin A or vitamin C."

8. Vitamins and Anaphylaxis

The anaphylactic reaction of the animal to foreign proteins is of the antigen-antibody type, in this respect being similar to an immunity reaction. Perhaps the first suggestion that vitamins may be involved in this reaction was the publication of Abderhalden and Wertheimer (135) reporting that pigeons fed upon a polished rice diet are more sensitive to anaphylactic shock than are pigeons on a normal diet. Wedgewood (136) later showed that the natural resistance of the rat to anaphylaxis could be dispelled by maintaining the animal on a diet deficient in water-soluble vitamins. To what extent general undernutrition entered into the picture in these two studies cannot be surmised. Other workers have observed an amelioration of the symptoms of shock if vitamin B_1 is injected between the sensitizing and the shock dose of foreign protein (137, 138). With reference to vitamin C, the evidence is quite confusing, ranging from strongly positive (139), in which complete protection of guinea pigs against the lethal effects of anaphylaxis was afforded by injections of lemon juice; through less positive (140), in which partial protection was afforded if a large dose of the vitamin is injected simultaneously with the sensitizing dose or shortly before; indeterminate (141), in which complete inhibition of hypersensitive manifestations was secured in only 2 out of 16 guinea pigs by the daily parenteral administration of ascorbic acid during the period of active sensitization; to frankly negative (142, 143, 144, 145), in which no appreciable effects of ascorbic acid on anaphylaxis were detected. The problem evidently needs further study by a technic that avoids the uncertainties evidently inherent in some or all of the investigations cited above.

It has apparently been the hope of many workers that the study of the experimental problem of anaphylactic (or protein) shock would afford some clue to the solution or management of the clinical problem of allergy, at least in so far as allergy involves protein sensitization. Shaw and Thelander (146) visualize the essentials of allergic phenomena as follows: "The capacity for allergic sensitization and subsequent shock phenomena is fairly universal; under ordinary conditions it is spontaneously exhibited pigs, showed a progressive decrease in liver esterase activity during the development of the deficiency, and a marked drop in succinic dehydrogenase activity of heart and skeletal muscle. It is interesting to compare the latter effect with analogous events in riboflavin deficiency (72), in which the succinic dehydrogenase activity of the liver, but not of the heart and skeletal muscle, was depressed. In such experiments as these, in which dietary conditions are imposed that ultimately impair the appetite of the experimental animals, it would seem wise to test the effect of dietary restriction alone (undernutrition) on enzyme concentration, since it has been shown that such an effect may eventuate (72). King (122) is inclined to the view that ascorbic acid in animal tissues acts "as a regulating and protective agent, perhaps exerting an effect upon other more effective hydrogen carriers and other important enzyme systems, rather than functioning as a major carrier itself."

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9. Vitamins and the Metabolism of Hemoglobin

The relation of vitamins, whether direct or indirect, to the synthesis and degradation of hemoglobin is but another instance of the many associations of these micronutrients with amino acid aggregates so largely contributing to the structure and functioning of the tissues. Aron (150) observed that adult guinea pigs during the development of scurvy exhibit a mild hypochromic anemia, which is cured, sometimes with dramatic suddenness, by administration of ascorbic acid. The young guinea pig usually succumbs before anemia develops. That this effect of ascorbic acid on hemoglobin formation may be quite indirect is indicated by investigations of the relationship of ascorbic acid to cobalt polycythemia. Barron and Barron (151) have shown that the daily intravenous injection of ascorbic acid into rabbits in a condition of cobalt polycythemia depresses the erythrocyte count, or, if administered simultaneously with cobalt, ascorbic acid prevents entirely the appearance of polycythemia. They also demonstrated that cobalt depresses the rate of respiration of the immature red cells present in the blood from polycythemic rabbits, and they visualize the etiology of cobalt polycythemia as being a direct result of this depression exerted in the hematopoietic tissues. The inhibition of the respiratory function of immature red cells in the bone marrow leads to their early release into the systemic circulation. Davis (152) later reported that cobalt administration to dogs, and anoxia, show additive effects in the production of a marked polycythemia. Ascorbic acid administration depresses the cobalt effect but not the polycythemia induced by exposure to low atmospheric pressure. Cobalt diminishes the ascorbic acid content of the blood from dogs with cobalt polycythemia. On the basis of his results, Davis suggests that cobalt may stimulate erythropoiesis (and hemoglobin formation) by interfering with a respiratory function of ascorbic acid. From another view point, it seems reasonable to concur with Barron and Barron in the conclusion that "ascorbic acid seems to assist in the maintenance of a determined level of red cells in the circulating blood."

The participation of riboflavin (153) and pyridoxin (154) in hemoglobin regeneration in dogs and rabbits, respectively, following blood withdrawal has been reported. Vitamin D may also be involved under certain conditions (155), apparently by reason of its effect upon calcium and phosphorus metabolism, which in turn modifies favorably or unfavorably the utilization of iron. Evidently the conditions governing hemoglobin formation and regeneration are complex and involve vitamins only indirectly.

In the physiological breakdown of hemoglobin, the porphin ring is ruptured in the alpha position and bile pigments are ultimately formed. In a series of important articles by Lemberg, Legge, and Lockwood (156–160, incl.) it was shown that *in vitro*, but under physiological conditions with reference to temperature, pH, and concentrations of the reacting substances, there is a coupled oxidation of ascorbic acid and hemoglobin, the rate of which was increased by glutathione. The reaction leads to the formation of a bile pigment-hemoglobin (choleglobin), while prolonged action of ascorbic acid on choleglobin removes the bound iron and sets free biliverdin. Vestling (161) has demonstrated one step in the cycle postulated by Lemberg, Legge, and Lockwood for the formation of bile pigments from hemoglobin by showing that, in accordance with thermodynamic prediction, ascorbic acid at pH 7.0 and 0°F. will reduce methemoglobin to the extent of 80 to 90 per cent within 6 hours. If these reactions occur *in vivo*, ascorbic acid assumes an important rôle in hemoglobin catabolism.

Under certain abnormal conditions, the catabolism of hemoglobin proceeds, initially at least, without rupture of the porphin ring with the production of porphyrins (162) and their excretion in the urine. Among these conditions is the deficiency disease, pellagra, preventable and curable by the administration of nicotinic acid. The light sensitivity of pellagrins and the severity of skin and mucous membrane lesions have been correlated (163) with the porphyrin excretion in the urine. Porphyrinuria is a frequent (164, 165), though not a universal (166, 167), concomitant of pellagra. When it does occur, it may be decreased by dosage with nicotinic acid (164, 165). Furthermore, porphyrinuria associated with diseases other than pellagra is promptly decreased following nicotinic acid administrations (164). Deficiency of nicotinic acid in the diet may evidently be involved in an abnormal type of hemoglobin degradation in the body, but probably indirectly through some damage to liver function.

There is also some evidence that pantothenic acid is also involved in porphyrin production (168), since in the absence of this vitamin from the diet, rats secrete protoporphyrin and coproporphyrin from the Harderian glands, the secretion passing by way of the naso-lacrimal duct to the nose of the animal where it appears as a red deposit on nose and whiskers.

10. Vitamin K and Prothrombin

In avitaminosis K, induced either by the failure of its synthesis by the bacteria of the intestinal tract, the excessive loss of such synthesized vitamin in the feces, as following mineral oil administration, its failure of absorption, as in jaundiced or bile-fistula animals, or its absence from the diet, the prothrombin level of the blood falls, the coagulation time is greatly prolonged and a hemorrhagic disease develops, in which animals may bleed to death from wounds (such as that produced by plucking the feather from a bird) that would be insignificant in the normal animal. This effect of a deficiency of vitamin K seems clearly to be mediated through the liver, since hepatic injury alone will produce hypoprothrombinemia, and in the presence of a sufficiently severe hepatic necrosis, administration of vitamin K cannot correct the condition (169). It has been proposed that vitamin K, and vitamin K analogs with suitable reduction potentials and side chains, may influence the action of liver cathepsin in favor of the synthesis of prothrombin (170).

The purification of prothrombin and thrombin (171, 172) reveals that they are both proteins containing small percentages of carbohydrate, and that prothrombin exhibits electrophoretic properties which place it in the upper globulin fraction of the blood proteins.

It is claimed that nicotinic acid possesses blood coagulation properties (173), possibly by promoting the formation of more prothrombin or of less antithrombin, or possibly merely through its hemolytic action, and the release of thromboplastin (another protein) from the disrupted elements of the blood. Mention may also be made of the claim (175) that ascorbic acid and calcium enhance the coagulating properties of histidine in some unknown manner.

V. Specific Physiological Relationships Between Vitamins and Amino-Acidogenic Hormones

An immense literature has accumulated concerned with relationships between those hormones presumably derivable from amino acids, particularly thyroxin and adrenalin, both being metabolic derivations of tyrosine or phenylalanine, and the various vitamins. A more complete discussion of the very confusing picture presented by these contributions is gladly left to the other chapters in this volume where it more properly belongs. The following discussion will mention briefly a few illustrative examples of the many relationships that may exist.

1. Thyroxin and Vitamins

The requirement of dogs for the vitamins of the B complex, but probably vitamin B_1, is definitely increased by experimental hyperthyroidism (176), as evidenced by the fact that on a B deficient diet anorexia appeared in one-half to two-thirds the time required during the control period. The quantity of food ingested voluntarily in the control (normal thyroid) and in the hyperthyroid periods was approximately the same. That the increased requirement of vitamin B_1 was associated with the increased intake of food and not with the increased metabolic rate induced by hyperthyroidism is shown by the fact that a similar experiment with dinitrophenol (177), in which an increase in metabolic rate occurred but no increase in voluntary food intake, gave no evidence of an increased requirement of the B vitamins. The increased expenditure of vitamin B_1 and increased losses from the body induced by thyroid administration have been clearly demonstrated by tissue analysis (178, 181), and measurements of urinary output (179). The loss of appetitie (180) and loss of weight (181) characteristic of hyperthyroid animals are not direct results of this condition, but are directly traceable to depletion of vitamin B_1 stores, and may be prevented by vitamin administration. In this sense there is an antagonism between thyroxin and vitamin B_1. On the other hand, it has been reported that thyroidectomy inhibits the storage of vitamin A in the liver (182), while thyroxin administration promotes storage, or regulates it.

The increase in basal metabolism that follows the injection of thyrotropic hormone or of thyroxin is diminished appreciably by simultaneous injection of vitamin B_1 (183), vitamin A or carotene (184, 185), or vitamin C (185). These vitamins alone, in the moderate doses exhibiting these associative effects, have no appreciable effect on the basal metabolic rate.

There are also reports indicating disturbances in riboflavin metabolism (186) and ascorbic acid metabolism (187, 188), of which the literature citations are more or less random selections.

2. Adrenaline and Vitamins

The high concentration of ascorbic acid in the adrenal gland, particularly the cortex, suggests an association between the activity of the gland and the metabolism of the vitamin. It is claimed that stimulation of the adrenal gland causes a fall in its content of ascorbic acid (189), while under certain conditions ascorbic acid has been observed to augment the effect of adrenaline (190, 191). Intravenous injections of adrenaline into guinea pigs in a condition of hypovitaminosis C have little or no effect on blood pressure unless accompanied by simultaneous dosages of ascorbic acid (192). Other investigations have minimized (193) or negated (194) any essential or intimate relationship between adrenaline and ascorbic acid. Relationships between adrenaline and vitamin B_1 (195) and riboflavin (196) have been reported.

Again the reviewer admits that this is not a critical discussion of the relationships between vitamin and proteinogenous hormones. Possibilities, selected more or less at random from the mass of published material, have been cited as indicative, but not neceesarily demonstrative, of the relationships claimed. For a critical appraisal of these possibilities the reader is referred to other chapters where similar references are made.

VI. Specific Physiological Relationships Between Vitamins, Amino Acids and Their Simple Derivatives

1. *Ascorbic Acid and Glutathione*

Both ascorbic acid and the tripeptide, glutathione, are highly reducing substances. In animal tissues, they both occur in the reduced and an oxidized form, and the reversibility under physiological conditions of the initial steps in their oxidation has been abundantly proven. *In vitro*, the further degradation of dehydroascorbic acid at pH 7.4 and 37°C. proceeds rapidly to the production of products possessing no antiscorbutic action, that is, the degradation of dehydroascorbic acid is an irreversible process (197). *In vivo,* the dehydroascorbic acid is protected from these irreversible processes, probably by reduction to ascorbic acid. That the protective agent is glutathione. is suggested by the observation of Szent-Györgyi that ascorbic acid oxidase does not oxidize ascorbic acid in the presence of glutathione. Instead, oxidation of glutathione occurs with reduction of the enzymatically produced dehydroascorbic acid. Only when glutathione, GSH, has practically disappeared does the oxidation of ascorbic acid proceed. This protective action of glutathione on ascorbic acid has been confirmed by Borsook and coworkers (197) and by Hopkins and Morgan (198), who found that this action of glutathione extends also to the oxidation of ascorbic acid by copper catalysis. The latter workers reported the presence in cauliflower juice of an enzyme that catalyzes the reduction of dehydroascorbic acid by glutathione and thus greatly enhances the protective action of glutathione towards ascorbic acid. Kertesz (199) was unable to obtain evidence for the existence of a catalyzing effect of cucumber or cauliflower juice on the reduction of dehydroascorbic acid by glutathione. However, Crook and Hopkins (200) were able to confirm the experiments of Hopkins and Morgan, particularly with reference to the existence of the enzyme catalyzing the reduction of dehydroascorbic acid in certain plant juices, and later Crook (201) succeeded in separating this enzyme from ascorbic acid oxidase.

The work of Borsook and others (197), indicating that glutathione was mainly responsible for the reduction of dehydroascorbic acid in animal tissues was confirmed by Schultze, Stotz, and King (202), who showed further that acid-insoluble —SH compounds have a similar effect. However, both the latter paper and a companion paper from the same laboratory (203) cast some doubt on the normal rôle of glutathione in reducing small amounts of dehydroascorbic acid in the presence of an excess of ascorbic acid, to complete a major respiratory cycle.

2. Vitamins and Creatine Metabolism

The derivation of creatine in animal metabolism from amino acid precursors has been fairly clearly indicated by biochemical research of the last few years. The effective experiments of Bloch and Schoenheimer (204), in conjunction with research observations from other laboratories, pictures the biogenesis of creatine somewhat as follows: "Glycine reacts with the amidine group of arginine to form guanidoacetic acid, and this is methylated to form creatine by shift of the methyl group from methionine." The high concentration of creatine in the muscle tissue and its involvement as phosphocreatine in the reactions concerned with the liberation of energy for muscular contraction, even though the significance of the involvement is not clear, would lead one to expect that any derangement of muscular function would be reflected either in the creatine content of the muscle or in the excretion of creatinine or creatine in the urine.

When vitamin E, α-tocopherol, is absent from the diet of an animal, either by reason of the selection of food materials, or of its destruction by oxidation, or when its absorption is impaired or inhibited, as in bile fistula dogs (205), a nutritional muscular dystrophy will eventually develop, evidenced by a decreased maximal contractile power. As might be expected, microscopical lesions in the muscles develop before functional impairment is evident to casual observation (206). The biochemical changes in the muscle include a decrease in the creatine content (207, 208) and in the content of phosphocreatine (209). Creatine appears in the urine in considerable amounts, irrespective of undernutrition (210), and with no disturbance in the excretion of creatinine. Evidently the rate of dehydration of creatine in the normal endogeneous catabolism is not appreciably disturbed, although the expulsion of creatine as such from the muscle and its appearance in the urine represents an evident disturbance in its metabolism.

The administration of α-tocopherol in adequate amounts by mouth relieves the clinical symptoms (211, 212, 213), raises to normal the concentration of creatine in the muscle and dispels the creatinuria (210, 211).

It is evident that α-tocoperol is intimately involved in creatine metabolism, but whether it is directly or indirectly involved is impossible to say. Parenteral administration of the vitamin is not particularly effective unless it is administered in a water-soluble form, such as the sodium phosphate of α-tocopherol (214).

There are reports of the implication of other vitamins in creatine metabolism, such as the removal of an experimentally induced thyroxin creatinuria in human subjects (215) by large doses of ascorbic acid, a marked increase in the excretion of creatine and creatinine in a child with glycogen disease by saturation with ascorbic acid (216), and a reduction and sometimes a disappearance of physiological creatinuria in normal infants by administration of vitamin B_1 in daily doses of 0.5 mg. per kg. of body weight (217). Beard and Pizzolato (218) reported the results on the excretion of creatine and creatinine by normal adult rats of the injection of a series of vitamins. In most cases, the variations in output were so irregular or so slight as not to justify a conclusion. The injection of α-tocopherol seemed to depress the excretion of creatinine and to increase the excretion of creatine, interpreted by the authors as indicative of a transformation of creatinine into creatine, although Bloch and Schoenheimer, using the isotopic nitrogen technic, could not demonstrate the reversibility in rats of the normal transformation of creatine into creatinine (219).

3. Vitamins and Amino Acid Metabolism

The functions of choline are discussed elsewhere in this volume, but as they relate to amino acids they are properly included in this chapter. It was shown by du Vigneaud and coworkers (220) that homocystine or homocysteine is incapable of supporting growth in rats on a diet devoid of methionine and cystine and containing ryzamin-B as a vitamin concentrate. When tikitiki extract and milk vitamin concentrate were used in place of ryzamin-B, growth resulted, indicating the presence of a factor or factors in the latter, but not in the former, vitamin concentrate that permitted the utilization of homocystine. This factor was identified as choline in a later paper from the same laboratory (221), in which betaine was also shown to be effective, but not so effective as choline (222). The effectiveness of choline in promoting the methylation of homocysteine to produce methionine was later confirmed by Welch (223) for rats and by Klose and Almquist (224) for growing chicks.

The production of fatty livers in rats on a low-protein, high-fat diet and its prevention or cure by choline indicate the effective lipotropic activity of this vitamin. Cystine had previously been observed to cause extreme fatty infiltration of the liver (see 225 for literature citations), a finding later abundantly confirmed. Methionine, on the other hand, reacted similarly to choline. The antagonistic effects of cystine and choline and the similar effects of choline and methionine were further studied by Channon and others (226), who assessed the lipotropic effect of methionine at about one twelfth that of choline. The lipotropic effects of proteins seem to depend largely, though not necessarily entirely (227), upon the proportion of methionine and cystine contained in them. Treadwell, Groothuis, and Eckstein (228) obtained results not inconsistent with the conclusion that methionine in the protein molecule is about as effective as free methionine in lipotropic effect. In interpreting the lipotropic effect of choline one naturally associates the fat-removal effect with the occurrence of choline in phospholipids, and the function of the latter compounds in fat transport. In fact, Perlman and Chaikoff (229) have shown that choline speeds up phospholipid metabolism in the liver. So also does methionine, but so also does cystine (230), which antagonizes the lipotropic effects of choline and methionine. Evidently the phenomenon of lipotropism in the liver has not been solved. Cirrhosis of the liver may also be produced on low-choline diets and be alleviated by choline, methionine or casein (231, 232, 233).

In a series of papers (see 225), Griffith and his collaborators have studied a characteristic hemorrhagic degeneration of the kidneys produced in young rats on choline deficient diets. These young rats, 21 to 26 days of age, also developed fatty livers, ocular hemorrhages, splenic hypertrophy, a regression of the thymus gland and a marked elevation of the non-protein nitrogen of the blood. The remarkable effect of choline in curing these conditions was clearly shown, the curative effect resulting from the administration of amounts too small to influence the deposition of liver fat (234). As in the production of fatty livers, so in the development of hemorrhagic degeneration of the kidneys, methionine exerts an effect similar to choline, while cystine antagonizes the action of both (235). Proteins participate in this kidney-damaging effect in proportion to their contents of methionine and cystine (236), while other vitamins than choline are without effect (237). From the fact that betaine, like methionine, may substitute for choline in curing and preventing the condition, Griffith and his coworkers conclude (238) that the condition is the result of a dietary deficiency of the labile methyl supply. Cysteine, homocystine, and glutathione, as well as cystine, aggravate the condition. The latter effect is not, according to Griffith (225) a true antagonistic effect, but is an indirect result of the supplementing effect of these compounds on the casein of the basal diet, increasing the metabolism of the rat, the rate of growth and hence the requirement for choline. However, the effect of cystine is produced on diets containing edestin (239) and arachin (240), proteins whose limiting amino acid is not cystine, and also at levels of casein intake so low that a supplementary effect of cystine would be inconsiderable.

Not all of the nutritive effects of choline are reproduced by methionine, since Jukes (241) was unable to demonstrate in chicks any curative effect of methionine on perosis resulting from the consumption of a choline deficient diet, although choline supplements were effective, a finding that has been confirmed (242) on chicks and on turkeys (243). An associative effect of choline and cystine in the protection of rats against hepatic injury following the ingestion of "butter yellow," has been reported (244); choline alone was ineffective, while cystine alone actually aggravated the injury.

A possible relationship between sulfhydryl compounds and vitamin B_2 deficiency in rats was suggested by experiments reported by Itter, Orent, and McCollum (245), but could not be confirmed by Prunty and Roscoe (246), and was evidently a complication of the vitamin deficiency imposed by the use of a sulfhydryl-deficient basal diet. A possible relationship between nicotinic acid and the metabolism of sulfhydryl compounds is suggested by the low cystine content of the fingernails of pellagrins, unaccompanied by a change in the total protein content, during the prevalence of florid dermatitis (247). With the subsidence of skin symptoms and improvement in clinical condition, the cystine content of the fingernails returned to normal values.

A relationship between nicotinic acid and glycine is suggested by the fact that both in man (248) and in the dog (249) nicotinuric acid is one of the main end products of nicotinic acid metabolism. In neither subject, however, does the ingestion of glycine modify the proportion of the nicotinic acid excreted in the conjugated form. Nicotinuric acid does not exert an appreciable anti-blacktongue, and presumably antipellagric, potency (250).

Albanese and Buschke (251) have observed that a dietary deficiency of tryptophan in growing rats produces an epitheliodystrophic cataract that resembles most closely the cataracts encountered in riboflavin deficiency. Furthermore, vascularization of the cornea, with characteristic superficial capillary loops, developed in the majority of the tryptophan-deficient rats, both adult and growing, and could be at least partially reversed by tryptophan administration. The authors conclude: "Although the changes in tryptophan deficiency are not identical with those seen in other conditions [including ariboflavinosis] causing epitheliodystrophic cataracts, the similarity of the pathological picture is striking and suggests that some common metabolic path is interrupted in these disturbances."

There are other isolated indications of effects of vitamins on amino acid metabolism, among which may be mentioned an impairment in the oxidation of α-keto acids in vitamin B_1 deficiency (252), an effect probably similar to one previously noted (108); an involvement of ascorbic acid in urea production in the liver (253), probably associated with the augmenting effect of the vitamin on arginase (111); a retardation of transamination of amino acids in avian polyneuritis (254); and a participation of riboflavin in the oxidation of alanine by rat liver preparations (255), quite probably via the flavin-adenine-dinucleotide.

A number of chemical reactions between ascorbic acid and amino acids have been observed *in vitro*, and studied obviously in the hope that their counterparts would be found to occur *in vivo* and would aid in elucidating the processes of amino acid metabolism. Thus, ascorbic acid or dehydroascorbic acid have been found capable of dehydrating leucine (256) with the formation of NH_3 and strongly reducing volatile substances, and, in the presence of iron and oxygen, of deaminizing and decarboxylating a series of amino acids (257); in the presence of ultraviolet light, ascorbic acid is reported to promote the actinic transformation of tyrosine into dopa (258).

The reaction between ascorbic acid and histidine has inspired a number of investigations. In the presence of histidine, alkaline solutions of ascorbic acid turn violet and the color deepens over a period of several days to a brown color from the surface downward, other amino acids giving nonspecific yellow colors. Ammonia is one of the products of the reaction, which has been proposed by Abderhalden (259) as a specific test for histidine. Edlbacher and Segesser (260) have found that the reaction is best carried out in a buffered solution, that it is catalyzed by ferric sulfate, that it depends upon the presence of oxygen, and that it is given not only by histidine at ordinary temperatures but by all compounds containing the imidazole ring, being apparently specific for this ring structure. The reaction has been studied also by Holtz and coworkers (261).

According to Edlbacher and Segesser (260), an oxidative cleavage of the imidazole ring occurs and an oxidation product is formed which on alkalinization gives off a second nitrogen atom as ammonia. This interpretation of the reaction is confirmed by Greenblatt and Pecker (262), whose yield of ammonia from thioneine was so large as to indicate a cleavage of the imidazole ring. This interpretation harmonizes with Edlbacher's theory of the metabolism of histidine in the animal body, according to which the amino acid is absolutely resistant to oxidative deamination. Rather, it is broken down hydrolytically by the enzyme histidase, present in the livers of all higher animals. During this hydrolytic breakdown there is a cleavage of the imidazole ring with the production of optically active glutamic acid.

Under the conditions employed by Holtz and associates, histidine in the presence of oxygen is changed in part to histamine by ascorbic acid, more histamine arising from the *d*-histidine than from the *l*-histidine (263). The presence of iron in the solution stops or retards the formation of histamine (264). Besides histamine, there is formed one molecule of free ammonia and one molecule of ammonia that can be liberated by strong alkali. This decomposition of histidine is also promoted by sulfhydryl substances, such as glutathione and cysteine (265).

Some doubt has been cast by Greenblatt and Pecker (262) on the physiological significance of the ascorbic acid-histidine reaction in experiments upon human subjects, and upon rabbits and guinea pigs. Comparatively large amounts of ascorbic acid, administered either intravenously or intraperitoneally, leave the imidazole ring of thioneine untouched as revealed by blood analysis. Injected into human subjects, either alone or with ferric ammonium citrate, ascorbic acid gave no evidence in the subsequent response of the gastric glands to histamine, that it destroys histamine. However, it is conceivable that this somewhat (and necessarily) artificial testing of the thesis does not rule out the physiological rôle of the reaction between ascorbic acid and histidine.

The formation of complexes of dehydroascorbic acid with each of three sulfhydryl compounds, glutathione, cysteine, and thioglycolic acid, in acetic acid solution has been reported by Drake, Smythe, and King (266), the reactants combining in equimolecular quantities. The results of this experiment are interpreted as confirmation of the suggestion of Borsook and colleagues (197) that such complexes are formed in the reduction of dehydroascorbic acid by glutathione.

An interesting relationship between ascorbic acid and the metabolism of the aromatic amino acids, phenylalanine and tyrosine, has been revealed by the recent investigations of Levine and coworkers on infants and of Sealock and colleagues on guinea pigs and adult humans. An aberration in the metabolism of these amino acids was observed in premature infants receiving diets relatively high in protein (267). The defect is manifested by the appearance of *l*-*p*-hydroxyphenyllactic and *p*-hydroxyphenylpyruvic acids in the urine, with no appreciable amounts of homogentisic acid, even after the administration of single doses of as much as 2 g. per kg. of either tyrosine or phenylalanine. The administration of ascorbic acid completely eradicated the defect without necessarily raising the plasma ascorbic acid level (268). The administration of other vitamins, either singly or in combination, was without effect, contrary to the reported observation of Closs and Fölling (269) that such a defect occurs in vitamin B_1 deficiency in rats. Full-term infants fed similar high-protein diets fail to exhibit this defect in metabolism spontaneously, but the condition can be precipitated by the ingestion of a single dose of 1 g. of either phenylalanine or tyrosine per kg. of body weight, and can be removed by ascorbic acid ingestion. The occurrence of hydroxyphenyllactic acid and hydroxyphenylpyruvic acid in the urine of infants, either spontaneously, as in the immature infant, or after aromatic amino acid ingestion, as in the normal infant, may constitute a delicate criterion of ascorbic acid deficiency, or at least of a condition of low saturation of the tissues with the vitamin. Its value in adult nutrition may prove well worth investigating.

Sealock and others (270, 271) have obtained analogous results with guinea pigs subsisting upon a vitamin C deficient diet, but with this animal homogentisic acid as well as tyrosine metabolites appear in the urine subsequent to the administration of tyrosine or phenylalanine. The amount of tyrosine metabolites excreted represents a considerable proportion of the supplemental amino acid. The degree of vitamin C saturation in the tissues is an important factor in the production of the anomaly. This condition is a true experimental alcaptonuria in the guinea pig, and the excretion of homogentisic acid subsequent to a constant dose of *l*-tyrosine was proportional to the amount of ascorbic acid given (272). The condition may be obtained after the ingestion of *d*-tyrosine also, though not to the same extent, and the removal of homogentisic acid from the urine requires larger doses of ascorbic acid than are required when the alcaptonuria is induced by *l*-tyrosine feeding. Experimental alcaptonuria could be induced also in the human subject and could be prevented by ascorbic acid ingestion. However, clinical alcaptonuria in a human subject was quite refractory to ascorbic acid treatment (273), even to excessive doses of 1 to 4 g. daily.

The experiments just reviewed clearly indicate an intimate involvement of ascorbic acid in the metabolism of phenylalanine and tyrosine in animals for which the acid is a vitamin, and quite probably in those animals possessing the capacity of synthesizing ascorbic acid, evidently for some physiological function or functions.

VII. SUMMARY

The physiological relationships between vitamins and amino acids, their derivatives and aggregates, are largely either associative or indirect in character. Protein-vitamin combinations catalyze many important oxidation-reduction reactions, and the partnership of vitamin-containing coenzymes with enzymes (proteins) in hydrogen and phosphoric acid transfer facilitates many important metabolic transformations. These oxidation and phosphorylation reactions mediated by vitamin-protein relationships may involve the amino acids after they have been deaminized and thrown into the metabolic mixture, whose degradation furnishes energy for the maintenance of life; or they may involve amino acid derivatives in the performance of specific functions in the cells, exemplified by the phosphorylation and dephosphorylation of creatine in the muscle. Vitamin A, or A_2, in the form of conjugated carotenoid-proteins, constitutes an integral part of the retinal mechanism concerned with light detection and discrimination. Antigen-antibody reactions, in which the antagonistic relation of one protein to another may decide the fate of the entire animal organism, are apparently modified in a favorable way by some of the vitamins.

The relationship between the vitamins and the metabolism of hemoglobin, in favoring its regeneration and guiding it away from abnormal catabolic paths, is evidently an indirect one, in which the vitamins act on the cells and organs concerned with the formation and degradation of this respiratory protein. Similarly the relationship of vitamin K and the prothrombin of the blood is mediated through the liver.

The effects of vitamins on the physiological functions of the proteinogenous hormones illustrate another type of association or synergism between essential constituents of protoplasm, although the details of the picture are indistinct because of inconclusive experimentation, or interrupted because of incomplete elucidation.

There are, however, a number of direct contacts between vitamins and amino acids in metabolism that have either been demonstrated, as in the case of the methylation of homocysteine by choline to form methionine, or rendered probable by *in vitro* study, such as the protective action of the tripeptide, glutathione, on ascorbic acid. These direct contacts, either demonstrable or presumptive, are amazingly few.

Of all of the vitamins, the one most intimately and frequently involved in amino acid functioning and metabolism seems to be ascorbic acid, in so far as available information indicates. Its direct involvement in collagen formation seems well established. It seems to contribute to the maintenance of the normal level of hemoglobin in the blood, although apparently quite indirectly. In animal tissues, as King has said, it acts as a regulating and protecting agent on various enzyme systems, while it is itself protected from degradation by glutathione. Through a coupled oxidation with hemoglobin it may participate in its normal catabolism leading to the formation of the bile pigments. It apparently is concerned with urea formation in the liver, probably by exerting an activating effect upon arginase, though under certain conditions *in vitro* it seems capable of promoting deamination itself. Also *in vitro*, though not improbably *in vivo*, it is capable either of decarboxylating histidine with the production of histamine, or of cleaving the imidazole ring, depending upon conditions. Both of these reactions probably occur in animal metabolism, although the involvement of ascorbic acid is problematical. Ascorbic acid seems definitely to participate in the metabolism of aromatic amino acids, phenylalanine and tyrosine, since its absence from the tissues or its occurrence there in inadequate concentrations lends to an interruption of their normal metabolism and the excretion of their unoxidized catabolites or to the formation of an abnormal catabolite, homogentisic acid.

In spite of the many relationships and contacts between vitamins and amino acids in metabolism revealed by available information, and the many more yet to be revealed in all probability, it is surprising that a complete, or even a serious, breakdown in amino acid metabolism is not a characteristic feature of any avitaminosis, with the possible exception of avitaminosis C. Many experimental studies have shown that when the general effects of undernutrition are ruled out by proper means, the digestibility of dietary nitrogen is not appreciably disturbed by vitamin deficiencies, and the retention of nitrogen in the body may continue unaffected up to the onset of severe clinical symptoms, when all metabolic functions are impaired, directly or indirectly. With reference to the contacts between vitamin functions and amino acid functions in the animal body, there is safety in numbers. If one or two or three of these contacts is not properly implemented by the absence of one of the participating agents, a collapse of the entire structure of proteins, amino acids and amino-acid derivatives, almost coextensive with protoplasm itself, would seem inconceivable.

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The Photoreceptor Function of the Carotenoids and Vitamins A

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I. INTRODUCTION

Viewed against the background of devastation which vitamin A deficiency causes in the mammalian organism, night-blindness is a relatively minor symptom. From the same point of view the participation of vitamin A_1 in mammalian vision appears to be a peculiar digression from its principal physiological functions.

From another aspect however this relation assumes a different appearance. Within the entire range of living organisms, animal and plant, light-sensitive structures regularly, perhaps universally, contain carotenoids, the class of substances which includes the vitamins A. In this respect the appearance of vitamin A_1 in the mammalian retina continues an association which extends, so far as known, throughout the eyes of vertebrates and invertebrates and the photosensitive organs of multicellular plants, algae and fungi (76).

Is this a trivial association or has it some genuine significance? Of the many functions which have been attributed to carotenoids in plants the only one which at present appears to be both well supported and general in scope is a receptor role in photokinetic systems—those concerned with directed movements in response to light. This is a stimulating conclusion, for the experiments of Blaauw, Loeb, Hecht, Castle and others have made it plain that the physiological characteristics of photoreceptor systems are essentially parallel in plants, in the simply organized light receptors of lower invertebrates, and in the eye. Not only the presence of vitamin A_1 in the mammalian retina but its functional activity as well therefore has a counterpart of sorts in plants. It seems possible now that photoreceptor systems in general are linked in a common dependence upon carotenoids

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(7, 87); and that the continuity of this relationship provides a unique opportunity for tracing the functional evolution of the carotenoids and vitamins A.

The purpose of the present paper is to examine the validity of this concept and its implications as closely as present information permits. It is proposed to do this first by detailing the evidence for the presence of carotenoids in light-sensitive structures. Next various theories of the function of carotenoids in plants are discussed, principally to emphasize the unique significance of their photoreceptor function. The evidence for this function is next examined, in plants and in certain protistan forms. The thread of the argument is lost for want of information within the lower invertebrates. With the appearance of eyes in the arthropods and molluscs it is regained, and thereafter can be pursued in some detail.

II. Carotenoids in Light-Sensitive Structures

All photosynthetic cells appear to contain carotenoids. They are classic constituents of the chloroplast, taking precedence over the chlorophylls themselves since they occur in the chlorophyll-free plastids of etiolated, albinotic, and autumn leaves (97, 67). All types of algae contain carotenoids, brown algae and diatoms principally fucoxanthin (47, 96). Purple photosynthetic bacteria contain large quantities of highly characteristic carotenoids, principally rhodoviolascin—probably identical with the spirilloxanthin of van Niel and Smith (74, 73)—and rhodopin; and to a minor extent rhodopurpurin, rhodovibrin and flavorhodin. The common carotenoids of higher plants are almost entirely absent, though occasionally small amounts of β-carotene have been detected (38). Green sulfur bacteria were for a time believed to lack carotenoids entirely (72); Katz and Wassink (39) however have recently presented good evidence to the contrary.

Many of these photosynthetic structures exhibit photokinetic responses. Others which display kinetic reactions alone also contain carotenoids. Carotenoid hydrocarbons (carotenes) have been identified in the phototropic spore-bearer cells of the fungi *Phycomyces blakesleeanus* (62, 14) and *Pilobolus kleinii* (11). No other types of carotenoids occur in appreciable quantities in these cells. The etiolated coleoptile of the oat seedling (*Avena*), a classic object in the study of plant phototropism, contains both carotenes and xanthophylls, principally the latter; and following irradiation chlorophyll also appears (88, 11). Bünning (10, 11) has stressed the significant observation that in all these phototropic structures carotenoids are concentrated in or restricted to the light-sensitive zones.

The etiolated oat coleoptile looks completely colorless in gross. Its content of carotenoids warns against the ready inference that structures of this appearance necessarily lack pigments. On the contrary, of course, tissues can react only to those wavelengths which they absorb (the Grotthus-Draper Law). Those sensitive to visible light necessarily absorb in this region and hence are colored. If this is not apparent it can only mean that the pigmentation is dilute.

The possibility that carotenoids are associated with photosensitive structures in the lower invertebrates has not yet been explored; this at present constitutes the most serious gap in our information. With the first appearance of image-forming eyes in the molluscs and arthropods the thread of this association may be resumed. From here onward however the classic plant carotenoids and their simpler derivatives assume a secondary, accessory rôle as screening and filter pigments for the retinal cells. The active function in vision is pre-empted by a special group of derived carotenoids, the vitamins A. In spite of profound changes in the organization of retinas and in the form of individual receptor cells, all visual systems yet examined contain and depend directly for their function upon these substances.

III. Functions of Carotenoids in Plants

The position of carotenoids in the plant economy is not as yet clear. The lavishness with which these pigments are synthesized, their wide distribution, above all their intimate association with the chlorophylls, imply that they play some critical rôle in plant metabolism. This inference still for the most part lacks adequate support. No general view is yet available of the synthesis and destruction of the carotenoids themselves, or of their participation in the reactions of other plant constituents. Fundamental problems which Berzelius raised in 1837 relative to the formation of carotenoids in the leaf, the nature of autumnal yellowing, and the interconvertibility of carotenoids and chlorophylls remain unresolved and the subjects of active investigation.

Carotenoids and Redox Systems. Arnaud (1) first suggested that since carotene spontaneously absorbs up to one-quarter—by later measurements up to about one-third—its weight of oxygen in air yet remains intact in the leaf, it probably participates in some reversible oxidoreduction system in metabolism. This view appeared to derive circumstantial support from the researches of Willstätter and collaborators, and was for a time adopted by them (97). On the one hand it was shown that the paired chlorophylls a and b ($C_{55}H_{72}MgN_4O_5$ and $C_{55}H_{70}MgN_4O_6$) and the principal carotenoids of the leaf, carotene and xanthophyll ($C_{40}H_{56}$ and $C_{40}H_{56}O_2$), each differ by one mol equivalent of oxygen. This suggests that they might participate in a coupled oxidoreduction. Furthermore these pigments were found to maintain remarkably constant proportions in the plastids of a variety of green plants, again as though coupled in a mutual interaction.

To test this possibility Willstätter and Stoll (98) determined the concentrations of the chloroplast pigments under conditions which might be expected to unbalance their ratios: following long and intense irradiation, at high temperatures in light and darkness, and after treatment with narcotics. In no case was the ratio of the chlorophylls appreciably changed, and the same was true in general of the ratio of the carotenoids. During autumnal yellowing the chlorophylls maintained their normal proportions down to extremely low concentrations; the total carotenoid content of the leaf remained virtually unchanged, while the xanthophyll/carotene ratio in some instances remained constant, in others rose to as much as three times the normal value. In short, under various conditions of stress the concentrations and ratios of the chloroplast pigments either did not change at all, or altered in ways which in no way implied interconversions among the pigments or coupled interactions. It is possible that methods more specifically designed to trap fleeting variations and to guard against possible changes during extraction of the pigments might have yielded somewhat different results. In any case Willstätter and Stoll rejected the Arnaud hypothesis on the basis of these experiments; and no reliable evidence has since been produced that carotenoids participate in oxido-reduction systems in the plant or that they are mutually interconvertible by such reactions.

It now appears more likely that the maintenance of more or less constant proportions among the chloroplast pigments is related to their formation with protein of large molecular complexes into which they enter in roughly constant combining ratios (51). The existence of such complexes—so-called chloroplastins, phytochlorins, or photosynthins—is now well established; and evidence is accumulating that they represent the predominant state of chlorophylls and perhaps also of carotenoids in the living cell (50, 51, 24, 25, 39, 65).

Protection of Chlorophyll. Early workers on chlorophyll had frequently contrasted its great stability in the leaf with its rapid photo-oxidation in extracts. Willstätter and Stoll (1918) suggested that carotenoids, if they function at all in photosynthesis, might act to protect chlorophyll from decomposition. The point of this suggestion was removed with the discovery that unlike the chlorophylls themselves the chlorophyll-protein complexes of photosynthetic cells are highly stable in aqueous extracts kept in light or darkness (50, 39, 64). The carotenoids which are present in these complexes appear to be similarly stable; this probably explains the resistance of carotene to autoxidation in the leaf, the phenomenon which troubled Arnaud.

Interconversions of Carotenoids and Chlorophylls. In his pioneer paper on autumnal coloration Berzelius (5) suggested that the yellow pigment of the leaf ("xanthophyll") is formed by some change, induced by cold, in the same organic processes which otherwise synthesize leaf-green. He tried without success to convert the green into the yellow pigments and *vice versa.* This general type of idea gained definite point with the penetrating observation of Willstätter and Mieg (95) that their empirical formula for carotene, $C_{40}H_{56}$, suggested structural relationship with both the terpenes, $C_{10}H_{16}$, and with the alcohol of chlorophyll, phytol, $C_{20}H_{39} \cdot OH$. Later researches justified this forecast. All these substances are structurally derived from isoprene, C_5H_8. Aliphatic terpenes contain two such units, phytol four (hydrogenated), and carotenoids as many as eight. It is clear that interconversions between carotenoids and chlorophylls must be confined to the phytol residue of the latter pigments.

Rudolph (60) has examined the possibility that carotenoids contribute directly toward the synthesis of chlorophylls in bean seedlings (*Phaseolus multiflorus*). When etiolated seedlings are irradiated with red light the concentrations of chlorophylls a and b rise rapidly for 2–3 hours, while those of carotene and xanthophyll fall by roughly equivalent amounts. In blue light the concentrations of all these pigments rise initially, the chlorophylls comparatively slowly. In both red and blue light the later changes observed in these experiments are complicated, perhaps due to the opening of new channels of pigment production by photosynthesis. Rudolph has proposed that blue light promotes the formation of carotenoids from precursors in the etiolated plant, and that the carotenoids in turn act as precursors of phytol in the synthesis of chlorophylls. The transient fall of carotenoid concentration with rise of the chlorophylls has since been observed in etiolated bean, wheat and cress seedlings on irradiation with white light (63). These experiments offer some support, though indecisive, for the idea that carotenoids may contribute toward chlorophyll synthesis.

The converse idea—that the degradation of chlorophylls may provide material for carotenoid synthesis—has also been considered. It is a striking fact that in certain situations chlorophylls disappear simultaneously with the rapid formation of carotenoids. Kuhn and Brockmann (43) have studied the yellowing of the calyx of *Physalis*, the Chinese lantern plant, in which as chlorophyll vanishes zeaxanthin dipalmitate is formed. The green calyx was found to contain about 0.14–0.17 per cent phytol in chlorophyll (the only form in which it was estimated). In yellowing 0.6–1.0 per cent of zeaxanthin is produced. The initial content of chlorophyll phytol is therefore not nearly sufficient to supply the material for carotenoid synthesis.

This possibility has particular force in the case of lycopene, the principal carotenoid of the ripe tomato, which possesses the structure of two desaturated phytyl residues joined symmetrically end-to-end. It has in fact been shown that completely hydrogenated lycopene is identical with the product obtained by condensing two molecules of dihydro-phytol (36); and Karrer has suggested that a comparable condensation may account for lycopene synthesis in the plant.

Kuhn and Grundmann (44) attempted to test this view by measuring changes in tomato fruits during ripening. The green fruit contains less than 3 mg. of chlorophylls a and b, or less than 1 mg. of chlorophyll phytol per 100 g. of fresh tissue; as this is lost the ripe fruit gains about 7.75 mg. of lycopene in addition to small amounts of other carotenoids. Again much more carotenoid is formed than is accounted for by the initial supply of chlorophyll phytol.

Actually such negative information as this is scarcely pertinent to the problem. It leaves open the possibilities of a partial participation of phytol in carotenoid synthesis, of the use of other sources of phytol than chlorophyll, and of a continuous synthesis of phytol even as the concentration of chlorophyll declines. We are left with the gross realization that in certain instances the carotenoids and chlorophylls vary reciprocally in concentration, and no definite ground as yet for excluding the possibility that they may be bound directly in some genetic association.

Carotenoids and Photosynthesis. A number of ways in which carotenoids might play an indirect rôle in photosynthesis have already been mentioned. The present paragraphs are concerned with the more restricted possibility that light absorbed by the carotenoids is used in photosynthesis. A qualitative attack upon this question is based upon the fact that the absorption of light by carotenoids is concentrated in the blue-green to violet regions of the spectrum; while that of the chlorophylls is distributed more or less symmetrically between the violet and red. In a narrow region of the blue-green almost the entire absorption of light by the plastid pigments of green leaves is due to carotenoids; while in the yellow, orange, and red, absorption is due almost entirely to the chlorophylls (67) (cf. Fig. 1).

Experiments which seem to show that the region of the spectrum in which carotenoids absorb is almost wholly ineffective in photosynthesis are of long standing, though they were not interpreted in this sense until comparatively recently. Draper in 1844 (16) measured the photosynthetic production of oxygen by plant tissues in various regions of the solar spectrum. He found it to be high in the red to yellow-green, and negligible in the blue and violet. Draper checked this result in a simple manner by showing that the insertion of a filter of potassium dichromate solution between sunlight and green leaves, which virtually eliminates the blue and violet, decreases the rate of photosynthesis so little as to be masked frequently by the errors of measurement. This experiment has been repeated periodically since, by Sachs (61), Pfeffer (58) and most recently by Willstätter and Stoll (98) with essentially the same results. The last-named authors concluded from it explicitly that light absorbed by the carotenoids is not used in photosynthesis.[2]

For a long period Engelmann was principal authority for the contrary opinion. Engelmann (21, 22) had measured the effectiveness of various wavelengths of light in promoting photosynthesis in a variety of aquatic plants. His method depended upon the fact that when a spectrum is thrown upon a leaf or algal culture, motile aerobic bacteria congregate principally in those areas in which oxygen is formed most rapidly by photosynthesis. Engelmann observed that in green, blue-green, red and yellow-brown cells the photosynthetic maximum moves progressively toward shorter wavelengths, in crude accordance with the over-all absorption of light. He concluded that photosynthesis must be mediated by other pigments in addition to the chlorophylls, including specifically the carotenoids.

To establish either conclusion decisively much more complete and precise experiments than these are required. It is in fact necessary to determine (a) the efficiency of photosynthesis at various wavelengths in a spectrum of known energy content; (b) the total absorption of light by the cells at each wavelength, and the fraction of this absorption due to each of the cellular pigments; and (c) the quantum efficiency of the photosynthesis due to chlorophyll itself throughout the spectrum (18).

Warburg and Negelein (91) were the first to attempt to cope with these requirements in experiments on the green alga *Chlorella*. Suspensions of cells were employed so dense as to absorb almost the entire incident light. The chloroplast pigments were extracted and their total absorption and the proportions of it due to the green and yellow pigments estimated. The photosynthetic yield (cu.mm. oxygen produced per calory of energy absorbed) was determined in four regions of the spectrum. It declined regularly with decrease in wavelength from the red to the green. In blue light, however, in which about 30 per cent of the total absorption was due to carotenoids, the yield was disproportionately low. The superficial appearance of the data is as though carotenoid absorption were ineffective in photosynthesis. Calculations on this basis however indicated quantum efficiencies in blue light higher than elsewhere in the spectrum; and the authors preferred to conclude that light absorbed by the carotenoids is used in photosynthesis, though with low efficiency. In view of errors implicit in these experiments this conclusion is not forced by the data and must be regarded with reservation.

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[2] This experiment is in fact equivocal, for the chlorophylls as well as the carotenoids absorb strongly in the violet and blue and should have been screened by a dichromate filter. The failure of photosynthesis to decline sharply under these conditions may have been due to the fact that the sources employed were very poor in blue and violet radiation. In this case however the experiments show nothing whatever.

In the absorption spectrum of suspensions of the purple bacterium *Spirillum rubrum* the maxima due to carotenoids are clearly separated from those due to bacteriochlorophylls (23). French has shown that the numbers of carbon dioxide molecules absorbed in photosynthesis per quantum of incident light correspond at various wavelengths with absorption by the chlorophylls alone. Light absorbed by the carotenoids apparently is not used in photosynthesis in this organism.

Dutton and Manning (18) have studied this problem in the marine diatom *Nitzschia closterium*. The pigments were extracted and the absorptions due to chlorophyll a, fucoxanthin, and the remaining carotenoids determined separately. Incident and transmitted light intensities were measured directly, and the mols of oxygen evolved in photosynthesis per quantum of light absorbed (the quantum yield) computed over a wide range of wavelengths. In green and violet light, in which 40 to 50 percent of the total absorption is due to carotenoids, the quantum yield is virtually the same as in the red, where absorption is due almost entirely to chlorophyll a. In the blue-green, where 90 per cent or more of the total absorption appears to be due to carotenoids, the quantum yield is only about 25 per cent lower than in the red. If the quantum yield in the blue-green is computed on the basis of absorption due to chlorophyll alone, energetically impossible values are obtained. These experiments furnish the most convincing demonstration yet available that light absorbed by carotenoids—in this case principally fucoxanthin—is used in photosynthesis.

Emerson and Lewis (19) have performed a similar study in the blue-green alga *Chroococcus*. The pigments were fractionated and the absorptions due to chlorophyll a, phycocyanin and the total carotenoids measured separately. The sum of these was found to compare very well, after maxima had been shifted to correspond with those in the intact cell, with the total absorptions of cell suspensions and of water extracts of the cells. Apparently in this organism as in others previously examined chlorophyll and the carotenoids are bound in some water-soluble complex, probably with protein. The quantum yield of photosynthesis was measured between 420 and 700 mμ. It remained virtually constant from 700 mμ to about 560 mμ, where absorption is due almost entirely to chlorophyll and phycocyanin. In the region 510–460 mμ in which carotenoid absorption is high, the quantum yield went through a deep minimum, finally to rise again at low wavelengths where carotenoid absorption declines and chlorophyll again absorbs strongly. The effect is superficially as though chlorophyll and phycocyanin were about equally efficient in photosynthesis, the carotenoids not at all. Yet calculations on this basis yield even lower expected yields in the region of carotenoid absorption than are actually observed. It is concluded that light absorbed by the carotenoids of this organism may be very slightly effective in photosynthesis.

These recent experiments provide extraordinary justification for Engelmann's belief (21) that in brown and blue-green—he included also red—algae other pigments in addition to chlorophylls function photosynthetically. In only one instance however—the diatom *Nitzschia*—have carotenoids been shown to act with an efficiency at all comparable with that of the green pigments. In all other types of cells so far examined carotenoid absorption appears to be only very slightly or not at all effective in photosynthesis.

Photokinetic Responses. Poor as the wavelengths absorbed by carotenoids are in general for photosynthesis, they are pre-eminent in stimulating a variety of photokinetic responses in plants—phototropic bending, phototactic orientation of free-swimming forms, light-growth responses, and chloroplast migrations. This sharp divergence between the spectral regions most effective in photosynthesis and in phototropism was demonstrated very simply by Sachs (61). Sunlight filtered through saturated dichromate solution, though highly active photosynthetically, had no phototropic effect whatever on a variety of etiolated seedlings. Sunlight which had passed through a cupric ammonium filter produced only very feeble photosynthesis but was highly active phototropically. Engelmann (20, 21) later demonstrated this difference in greater detail in the green flagellate *Euglena viridis.* In the solar spectrum photosynthesis was maximal in the red, minimal in the green, and rose again slightly toward the violet. In a gas light spectrum phototactic orientation was negligible in the red and green, rose to a high maximum in the blue, then fell again in the violet. The spectral sensitivities are almost directly complementary in the two cases.

Actually such experiments are not accurately comparable with one another or with the underlying absorption data unless the relative energies of the spectral radiations employed are known. These first became available with Langley's invention of the bolometer in 1880 and his measurement in 1884 of the distribution of energy in the spectra of sunlight and of the carbon arc. It was with the aid of these data that Blaauw (6) evaluated his classic measurements on the spectral sensitivity of phototropic bending in the oat seedling (*Avena*) and in the unicellular spore-bearer of the mold *Phycomyces nitens*. In both organisms the sensitivity is maximal in blue light and falls off sharply in the green and violet.

Such a determination of spectral sensitivity is frequently called an action spectrum. It consists in the proper instance of the reciprocal at each wavelength of the energy required to elicit a constant response. Its form and position in the spectrum are intimately related to the absorption characteristics of the pigment which absorbs the effective light. Action spectra may however be distorted considerably in shape and somewhat in position by the presence of other pigments and by special physical arrangements within the cells. No *a priori* assurance exists, therefore, that an action spectrum represents accurately the absorption spectrum of any cellular constituent.

The phototropic action spectra measured by Blaauw and foreshadowed in the experiments of Sachs and Engelmann correspond unequivocally with the absorption of light by a yellow pigment. None of these authors appears to have drawn this inference explicitly. Bachmann and Bergann in 1930 (2), however, suggested that the action spectrum of *Avena* seedlings resembles the absorption of a "chromolipoid," *i.e.*, a carotenoid. This view has since been developed by Voerkel (75), Castle (14), and Bünning (10, 11, 12).

The carotenoid theory of phototropic excitation rests upon three major considerations: (1) The photosensitive structures contain these pigments; evidence for this has been reviewed above. (2) The action spectra for photokinetic responses correspond more or less specifically with the absorption spectra of carotenoids. (3) No others of the principal pigments found in these photosensitive structures possess comparable spectra.

The last point is illustrated in Fig. 1. The principal plant carotenoids, xanthophyll and β-carotene, and fucoxanthin in the brown algae and diatoms, possess absorption maxima in the blue and blue-green, falling steeply to negligible values in the green and more gradually in the violet. Chlorophylls a and b and bacteriochlorophyll possess absorption maxima in the violet, chlorophyll b in a region of high carotenoid absorption, the others at considerably lower wavelengths; but all these pigments have additional maxima in the orange and red, where carotenoid absorption is virtually nil. Protochlorophyll, reported to occur in etiolated seedlings, possesses maxima in the orange and yellow-green (60). Phycocyanin and phycoerythrin, water-soluble pigments of the blue-green and red algae, absorb principally in the orange to green, and minimally in the range 435–470 mμ where carotenoid absorption is maximal (68).

It is necessary to recognize that the positions of these spectra depend to a degree upon the media in which the pigments are dissolved and their physical and chemical condition. The spectra of the fat-soluble pigments lie at shortest wavelengths in true solution in hexane, methanol, or

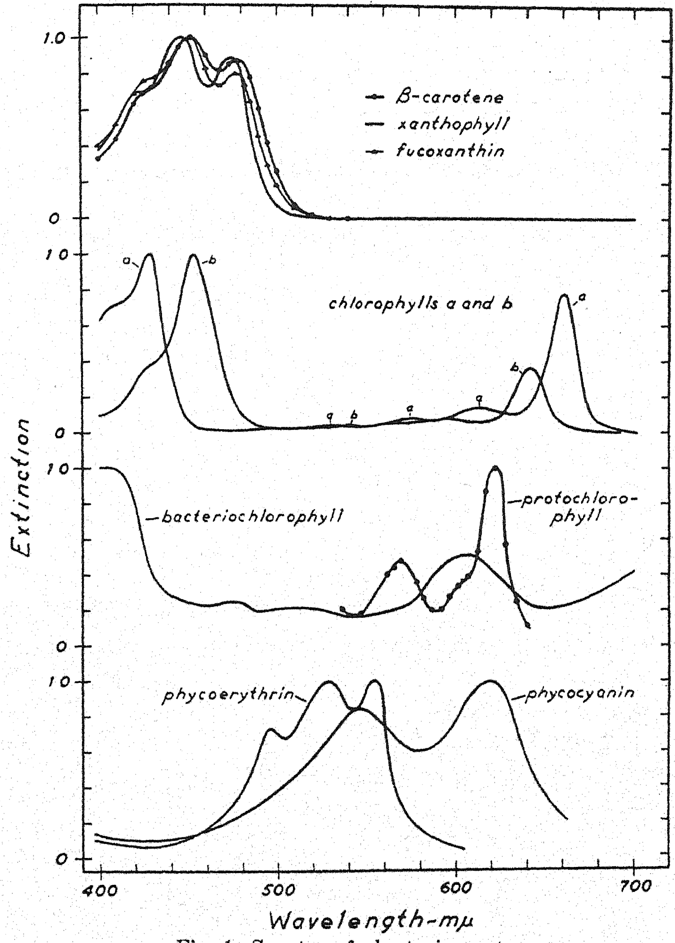

Fig. 1. Spectra of plant pigments

The carotenoids are in hexane solution (unpublished observations); chlorophylls a and b in ether (Haskin, 30a); protochlorophyll from the seed coats of *Cucurbita pepo* in ether (Rudolph, 60); bacteriochlorophyll from the purple bacterium *Spirillum rubrum* in methanol (French, 23); and phycocyanin and phycoerythrin from *Ceramium* in water (Svedberg and Katsurai, 68). The ordinates are extinctions, log I_0/I in which I_0 is the incident and I the transmitted intensity.

ethanol. They are displaced progressively toward the red in solvents of higher refractive index, in the extreme case carbon disulfide, in which the spectra lie some 30 mμ away from their positions in hexane or alcohol.

In ordinary plant oils the spectra of carotenoids are displaced about 12 mμ toward the red from their positions in hexane. In the living tissues the spectra of chlorophylls and carotenoids are ordinarily found 15–20 mμ toward the red from their positions in hexane or alcohol; this appears to be due primarily to the union of these pigments with protein, for the spectra of aqueous solutions of the pigment-protein complexes show a comparable displacement (50). These factors ordinarily exert more or less parallel effects upon the spectra of all the pigments in any given tissue, so that their relative positions as shown in Fig. 1 remain approximately intact.[3]

Even the gross character of carotenoid absorption therefore differentiates it from that of all other pigments present in considerable amount in the structures under consideration. This is an important conclusion, for it permits one to draw from experiments which show no more than a photokinetic sensitivity maximum in the blue, falling off toward the violet and negligible in the yellow and red, some assurance that one is dealing with a carotenoid.

Blaauw's measurements on *Avena* and *Phycomyces* yielded just this type of information. Both plants displayed broad action spectra, maximal at about 466 mμ in *Avena* and at about 495 mμ in *Phycomyces*, and falling off regularly toward both sides of the maximum. Castle (13) has since examined the spectral sensitivity of *Phycomyces blakesleeanus* and found a single broad maximum in the region of 440 mμ; this has been confirmed by Buder (9) and Bünning (12).[4]

A new development appeared in this field with the paper of Bachmann and Bergann (2). Just what it was these authors contributed has occasionally been misrepresented since, through no fault of their own. Bergann (3) had measured the light-growth and phototropic reactions of *Avena*, principally to demonstrate their essential parallelism. To isolate various regions of the spectrum he had used color filters of known energy transmission prepared by Bachmann. Bachmann and Bergann attempted to reconcile their light-growth measurements with those of Blaauw, in which highly monochromatic spectral light and a different response had been employed. Blaauw had performed two series of measurements on *Avena*, one in the carbon arc spectrum, showing a maximum at 466 mμ and ending with a lower value at 448 mμ; the other in a solar spectrum, showing a regular falling-off of sensitivity from 436 mμ into the ultra-violet. Each series was corrected separately for the relative energy distribution of the source; but Blaauw had no means of fitting both series together. He suggested tentatively that they be joined so that the solar series continue the decline in sensitivity already begun at the low wavelength end of the arc light series. Bachmann and Bergann, as the result of an involved computation of the energy relations within their own data, concluded that Blaauw's solar measurements should be multiplied by a factor of about 2.5 relative to the arc light series. The effect of this was to create a new and principal maximum, which does not appear in the direct measurements at all, at about 436 mμ. The total sensitivity curve as a result possesses two peaks, at 436 and 466 mμ, reminiscent of the principal bands of carotenoid absorption. Involved as the original basis of this conclusion was, it was soon confirmed in part by the measurements of Johnston (34) which revealed two maxima in the action spectrum of *Avena*, at about 440 and 472–475 mμ (Fig. 2).

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[3] This statement of course does not apply to phycocyanin and phycoerythrin, themselves water-soluble proteins of high molecular weight (about 300,000). The further structures of these pigments are still unknown.

[4] Haig (30) has measured the spectral sensitivity of *Avena* seedlings in which all but 1.5 mm. of the tip was shielded. He obtained an action spectrum maximal at 480 mμ and falling steeply to either side. The principal band of this spectrum is extraordinarily narrow, unlike any obtained previously with this material, and not related simply to the absorption spectrum of any of the known pigments of this tissue.

Bünning (10) has since obtained similar results with spore-bearers of the mold *Pilobolus kleinii*. The action spectrum for phototropic bending possesses two maxima, at about 445 and 485 mμ (Fig. 2). Bünning also measured photoelectrically the difference in absorption between pigmented and unpigmented zones of *Pilobolus*, and obtained in this way a spectrum of the total pigmentation *in situ* which agreed remarkably well with the observed action spectrum. He found the properties of the pigment in the cell and in extracts to be those of a carotene, apparently principally β-carotene.

A curious circumstance attends these two-peaked action spectra. It is obviously tempting to fit them directly to the spectra of carotenoid pigments. They are situated, however, peculiarly low in the spectrum for this. The bands of pure β-carotene in hexane lie at 451 and 482 mμ, those of pure xanthophyll at 447 and 476 mμ. This represents their extreme low-wavelength positions. Spectra of the crude pigments as extracted from tissues actually lie lower than this. In general as carotenoids are purified their spectra shift progressively toward a long-wavelength limit, much as the melting-point of an impure material is driven upward under comparable circumstances. It is possible that the physiological behavior of these pigments corresponds better with the contaminated state in which they occur in the tissues than with the pure crystalline condition.

Spectra of crude preparations of the total carotenoids of the oat coleoptile and of *Phycomyces*, dissolved in hexane, are shown in the upper portion of Fig. 2. The oat pigment possesses maxima at 442 and 470 mμ, that of *Phycomyces* at 449 and 475 mμ. Clearly they resemble in general form and position the action spectra from *Avena* and *Pilobolus*. Particularly striking are the parallel displacements of the absorption and the action spectra, due apparently to the fact that the principal carotenoid of the oat coleóptile is xanthophyll, that of *Pilobolus* and *Phycomyces* is β-carotene.

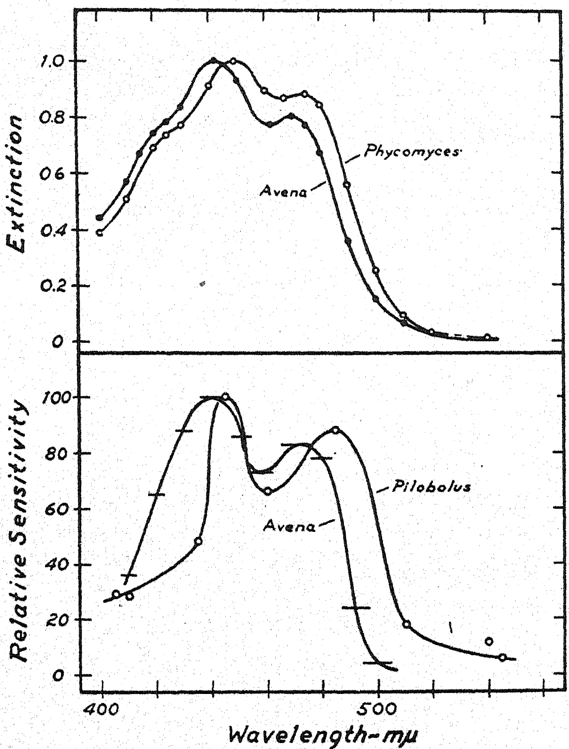

Fig. 2. Absorption spectra of carotenoids and action spectra for phototropic bending *Above:* Absorption spectra of the total carotenoids of the etiolated oat coleoptile (unpublished observations), and of sporangiophores of *Phycomyces* (14). *Below:* The spectral sensitivity of *Avena* (Johnston, 34) and of the sporangiophore of *Pilobolus*. (Bünning, 10). It is known that the pigments of *Phycomyces* and *Pilobolus* are virtually identical (11). The marked similarity in form of these absorption and action spectra supports the view that the latter depend upon the former. The difference in position of the absorption spectra reflects the fact that the principal pigment of *Phycomyces* is β-carotene, that of *Avena* xanthophyll. The parallel displacement of the action spectra of *Pilobolus* and *Avena* is added evidence that they are based upon the carotenoid absorptions.

It is difficult to avoid the conclusion that the action spectra should lie at appreciably *longer* wavelengths than the absorption spectra of the crude pigments in hexane. Actually however the principal maxima of both action spectra lie at *lower* wavelengths than those of the associated absorption spectra. The action spectrum of *Avena* proposed by Bachmann and Bergann lies as a whole much too low in the spectrum to be explained on any apparent basis. These puzzling discrepancies deserve further consideration.[5]

It would be mistaken to assume at present that any sufficiently careful series of measurements on phototropic bending should reveal a two-peaked action spectrum. Bünning (12) has been unable to find any evidence of a two-peaked sensitivity function either in *Phycomyces* or in immature spore-bearers of *Pilobolus*. This raises no special problems, since as pointed out above many accessory factors may distort an action spectrum out of all resemblance to its underlying absorption spectrum. Bünning (12) has discussed this matter in some detail.

Phototropic bending is not the only type of photokinetic reaction differentially sensitive to the wavelengths absorbed by carotenoids (14). Bottelier (8) has shown that protoplasmic streaming in the epidermal cells of the *Avena* coleoptile is inhibited maximally in the blue, the effect falling off in the violet and green and becoming negligible in the yellow. The general form and position of this function in the spectrum agree well with those established for phototropic bending. Voerkel (75) has measured the energies in broad spectral regions required to induce a constant state of chloroplast orientation in the moss *Funaria*. The sensitivity is maximal in the blue, falls off to low values in the green and is negligible in the red. In both these functions the absorption of light appears to be mediated by carotenoid pigments.

Phototropic bending is a consequence of differential growth in the coleoptiles of etiolated seedlings and in such unicellular structures as the *Phycomyces* sporangiophore (3, 94). It may be asked whether the effects of light on plant growth in general exhibit similar relationships to those discussed above.

Went (93) has recently examined the spectral sensitivity of a number of light-growth reactions in pea seedlings. Leaf growth is maximal in the red and orange; inhibition of stem growth is maximal in the red and yellow. Only the growth effect responsible for phototropic bending is maximal in the blue and negligible at long wavelengths, and may be ascribed to absorption of light by carotenoids.

[5] Bünning's measurements of the absorption spectrum of the pigment of *Pilobolus* in the living sporangiophore (10) agree very closely in form and position with the action spectrum for phototropic bending. It is difficult to understand, however, the circumstances which could bring the position of the main band of this pigment to shorter wavelengths in the tissue than in simple solution in hexane.

Weintraub and McAlister (92) have found the growth of the mesocotyl in *Avena* to be inhibited maximally in red light, at about 660 mμ. In the region 430–500 mμ where carotenoid absorption is high the sensitivity of this effect is minimal. This reaction clearly is not mediated through the carotenoid pigments.

Engelmann (20) has described a type of response to light radically different in nature from those discussed above. This is found in certain motile aerobic cells which either depend for their motility upon oxygen or are chemotactic to it. In situations in which oxygen is supplied primarily by photosynthesis, either of the cells in question or of associated plants, the action spectrum for motion or orientation corresponds with that for photosynthesis. Engelmann carefully distinguished such responses, which are really indirect chemotaxes, from the genuine "Lichtempfindungen". As type of the latter he cited the behavior of *Euglena viridis*, already mentioned above and discussed in greater detail in the section to follow. There is nothing to prevent a given organism from exhibiting both types of response—chemotaxis to oxygen with an action spectrum based upon chlorophyll absorption, and genuine phototaxis with an action spectrum based upon the carotenoids. This appears to be the case in certain purple bacteria (23).

Summary. Most of the functions heretofore attributed to carotenoids in plants still lack adequate demonstration. No evidence yet exists that they participate in reversible oxidoreduction systems in the plant, or that they exercise anti-oxidant functions in the tissues. Slight evidence exists that carotenoids and the phytol of chlorophyll may in certain instances be interconvertible. Light absorbed by the carotenoids has been shown to be used in photosynthesis with an efficiency approaching that of chlorophyll in the single instance of the diatom *Nitzschia*. In all other types of cells examined this activity is very slight or negligible.[6]

In a relatively small number, but wide variety, of plant structures the sensitivity of oriented reactions to light has been shown to be greatest in the blue region of the spectrum, less in the violet and negligible in the yellow to red. Carotenoids are the only known constituents of these structures which have absorption properties appropriate to such action spectra. In certain instances the form of the spectral sensitivity function (*Avena, Pilobolus*) displays details which can be related roughly to the form of carotenoid absorption spectra. In some structures also (*Avena, Pilobolus, Phycomyces*) carotenoid pigmentation has been shown to be concentrated in or restricted to the phototropically sensitive zones (Bünning). In all these cases it may be concluded tentatively that the primary process of photoreception is mediated through carotenoid pigments. This is as yet the only function in plants in which carotenoids appear to play a general and principal rôle.

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[6] Moewus (55) has reported remarkable effects of various compounds of the carotenoid acid crocetin on the behavior of *Chlamydomonas*. Crocin—the digentiobiose ester of crocetin—is stated to stimulate motility in *Chl. eugametos* in a concentration of 1:250 billion, or about one molecule per cell. Mixtures of the *cis* and *trans* stereoisomers of crocetin dimethyl ester in certain definite proportions are reported to activate the clumping of male and female cells which is prerequisite to their copulation. The chemotactic action upon which this phenomenon is based has also been demonstrated directly. Mixtures of the *cis* and *trans* compounds in precise proportions are said also to activate selectively various intermediate sexual types in this organism. The present author is in no position to review this work critically; see however the comments of Philip and Haldane (59), Thimann (69), and Murneek (56).

IV. PHOTOTACTIC SYSTEMS OF PROTISTA; ASTAXANTHIN

Under this heading I propose to discuss relations within a group of organisms which is included among both algae—as green flagellates and the volvocine line—and protozoa, as the subclass phytomastigina.

Ordinarily these organisms contain a structure which appears to be specifically concerned with photoreception, the stigma or eye-spot. This view has been disputed by Engelmann (20) who, from the behavior of *Euglena viridis* at the edges of sharp shadows, concluded that the light-sensitive zone is localized in the colorless region just anterior to the stigma. It is doubtful that any shadows to be obtained in the field of a microscope are sufficiently sharp-edged for such fine discrimination as this. Luntz (52) has re-examined this situation in *Chlamydomonas*, and found it impossible to localize the photosensitive area more closely than in the anterior third of the organism, which includes the eye-spot.

The action spectra for phototactic orientation of these organisms also indicate the stigma itself to be the organ of photoreception. Mast (54) has provided a very careful and detailed series of such measurements. White light projected at a right angle to narrow spectral bands from a monochromator was adjusted in intensity so as to induce a fixed angle of orientation in the organisms. The results were later corrected for the energy distribution of the source. The *Euglena* species examined all proved to be maximally sensitive in the blue, at 473–483 mμ (*Euglena viridis, gracilis, tripteris, granulata* and *minima*). Action spectra from *Phacus triqueter, Trachelomonas euchlora* and *Gonium* also were maximal in this region. That of *Chlamydomonas* lay further toward the red, at 504 mμ; while those of *Pandorina, Eudorina* and *Spondylomorum* were maximal in the green, at about 534 mμ. In all cases the sensitivity fell more or less symmetrically to both sides of the maximum. A selection from these data is shown in Fig. 3.

Laurens and Hooker (48) have measured the exposure times at various wavelengths of an equal energy spectrum needed to induce phototactic motion in a colony of *Volvox globatur*. The reciprocal of the exposure, a measure of stimulating effect, was maximal at about 494 mμ, falling symmetrically to either side. Since these measurements are based upon times rather than intensities, and the relation between time and intensity in stimulation is unknown, the form of this sensitivity function need not bear any simple relation to an absorption spectrum. Luntz (52) has reported short series of measurements of absolute threshold for phototactic orientation in *Eudorina elegans* and *Volvox minor*. In both organisms the sensitivity was greatest in the general region about 492 mμ, falling sharply to both sides of the maximum.

All these measurements agree in two general characteristics: the action spectra all possess single maxima, and are displaced considerably toward the red from those associated with phototropic bending (with the single exception of Blaauw's measurements on *Phycomyces nitens*, so far unconfirmed). A pigment possessing the absorption characteristics required by these action spectra should be orange, red or purplish in color, comparable therefore in external appearance with the pigment of the stigma.

The principal carotenoid of this type of organism has enjoyed a long history under a variety of names: the "haematochrome" of Cohn, "carotinin" of Zopf, and "euglenarhodon" of Tischer (57, 70, 71). It is apparently concentrated in the stigma and possibly dispersed throughout the other chromoplasts. It has recently been identified as astaxanthin (46). This carotenoid has not otherwise been found in any but animal tissues, where so far as now known it has had to be synthesized from plant carotenoids available in the diet. Its appearance in the protista adds a chemical ingredient to the already highly equivocal status of these organisms.

Spectra of astaxanthin published by Kuhn and Sörensen (45) which display three maxima are apparently in error. Tischer (70) could distinguish only a single band visually; and repeated photoelectric measurements in our laboratory have shown the spectra of astaxanthin and its esters to consist of a single broad band, almost identical in form with that of astacene, the familiar oxidation product of astaxanthin; but displaced toward shorter wavelengths 4–7 mμ, depending upon the solvent (Fig. 3). The astaxanthin spectrum lies considerably further toward the red than the main absorptions of the common plant carotenoids. In both form and general position therefore, it recalls the properties of the action spectra of protista as contrasted with those of phototropic plants.

How general the possession of astaxanthin is among protista is not yet known. It has been identified explicitly in *Haematococcus* and *Euglena*, in company with smaller amounts of the common plant carotenoids. Possibly more extended examination of this group of organisms will reveal the presence of other special pigments. Meanwhile it may be noted that the range of action spectra exhibited by the protista is not beyond the capacities of astaxanthin itself. In simple solution the maximum absorption of this pigment and its esters varies between about 470 mμ in hexane to about 492 mμ in pyridine. In ordinary cell oils its spectrum should agree reasonably well in position with the action spectra of *Euglenas* and various other flagellates (Fig. 3). Astaxanthin also readily forms complexes with protein which range in color from its native red to green, as in the ovoverdin of lobster eggs (66) and blue, as in lobster shells (46). Comparable complexes could easily meet the most extreme requirements of protistan action spectra.[7]

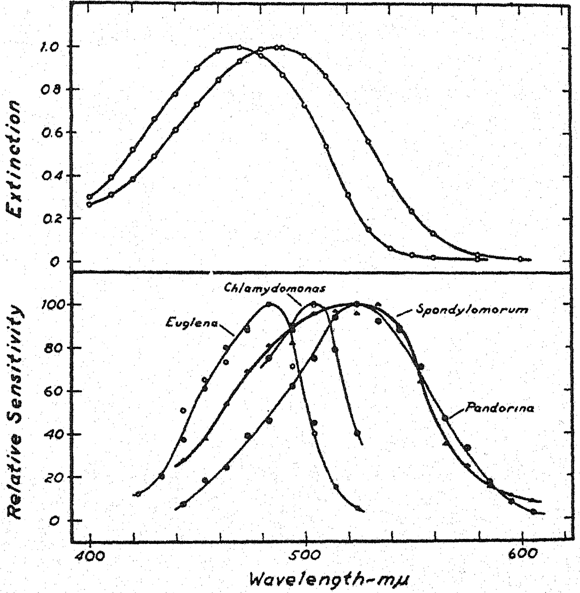

Fig. 3. The absorption spectrum of astaxanthin and phototactic action spectra of protista

Above: Spectra of astaxanthin dissolved in hexane (left) and in castor oil (right) (unpublished observations). *Below:* Spectral sensitivity for phototactic response in *Euglena viridis*, *Chlamydomonas globulosa*, *Spondylomorum quaternarium* and *Pandorina morum*, from Mast (54).

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[7] Luntz (52) has studied the phototactic sensitivity of a "colorless" protistan, *Chilomonas*. As noted above, to respond to visible light at all this organism must be able to absorb it, and hence must possess some pigmentation. Its colorless appearance may be taken as evidence that this is dilute. This conclusion is consistent with Luntz's observation that the threshold of *Chilomonas* to visible light is inordinately high, 750–1200 times those of colored protista examined. The spectral sensitivity was found to be roughly constant from the green to the violet, and rose to high values in the near ultra-violet (366 mμ).

V. Photokinetic Systems of Lower Invertebrates

To the degree that protista are admitted within this category we have already considered the most pertinent information which it contains. Photokinetic responses abound among the lower invertebrates; but few accurate measurements have been made of the associated action spectra, and nothing is known of the associated pigmentation.

In an attempt to establish the essential identity of photoreceptor arrangements in lower animals and in plants, Loeb and Wasteneys (49) repeated Blaauw's *Avena* experiments on newly formed polyps of the coelenterate *Eudendrium ramosum.* Lines of these organisms were exposed for various periods to the spectrum of the carbon arc, then replaced in darkness and the proportions which responded by bending observed. In the uncorrected spectrum the effect was sharply maximal in the blue at about 474 mμ, decreased in the violet, and was negligible in the orange and red. This result is almost identical with that of Blaauw; unfortunately we lack as yet the crucial demonstration that this material contains carotenoids.

In general the photic responses of lower invertebrates are pitched at longer wavelengths than those associated with phototropic bending, either of plants or of *Eudendrium.* They are in fact included roughly within the range of action spectra observed within the protista. Mast (54) found the phototactic sensitivities of the earthworm and of larvae of the marine worm *Arenicola* to be maximal at about 483 mμ, resembling in form and position those of the *Euglenas.* Hecht (31, 32) measured the relative energy in broad regions of the spectrum required to elicit the photic responses of the clams *Mya* and *Pholas* within a constant reaction time. The sensitivity of *Mya* is maximal in the blue-green, at about 490 mμ, falling steeply to both sides; that of *Pholas* in the green, at about 535 mμ, with possibly an added maximum in the ultra-violet.

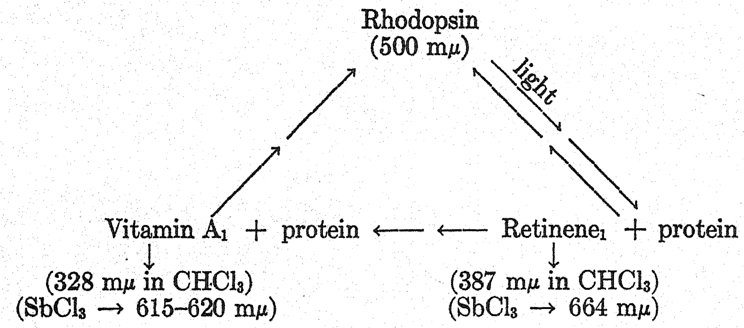
None of this information contributes materially to the problem under discussion. Before it can do so some systematic study must be made of the pigments of photosensitive structures in this great range of organisms. Such an exploration offers an opportunity to bridge what is now an enormous gap in the knowledge of photoreceptor systems. It promises in addition an insight into the genesis of the vitamins A. As the thread of the present argument is lost among the protista these substances have not yet appeared. When it is resumed in the retinas of the arthropods and molluscs vitamins A are present in high concentration and appear to dominate the photoreceptor process. Somewhere between these bridgeheads animals develop the capacity to degrade the molecules of plant carotenoids to yield the vitamins A. It should be important to locate the origin of this critical development.

VI. INVERTEBRATE AND VERTEBRATE EYES; THE VITAMINS A

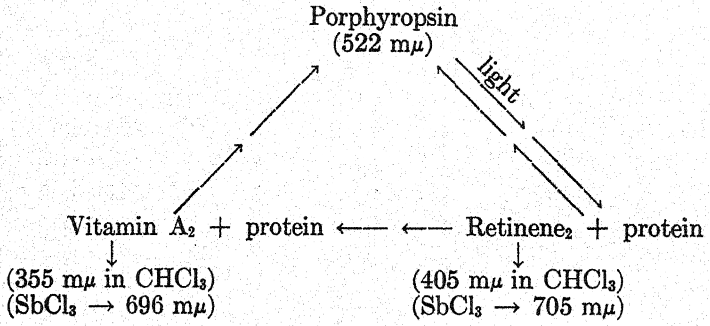
The first image-forming eyes are found in the arthropods and molluscs. Within these phyla they vary radically in construction. The only molluscan eye discussed below—that of the squid—contains a large double plano-convex lens and a retina composed of a single layer of cells, their photosensitive segments turned toward the light. The principal eyes of arthropods are of the compound or mosaic type, composed of a large number of independent units, optically isolated from one another by screening pigments, and each containing a complete dioptric apparatus. In vertebrates still a third type of structure is encountered, in which the retina is multi-layered and the receptor cells, which face away from the light, are of two kinds: rods, concerned primarily with responses to dim light; and cones, ordinarily the organs of vision in bright light and, at least in certain forms, color vision.

In spite of these wide differences in physical organization, the photoreceptor systems in all these eyes operate with a very limited group of chemical substances and a very restricted pattern of reactions. For this reason it will be expedient to digress briefly to describe the composition and arrangement of the visual systems which have been most thoroughly explored—those of the vertebrate rods. Two such systems are known, based upon the photosensitive pigments rhodopsin and porphyropsin, associated in retinal cycles with the vitamins A_1 and A_2.

Rhodopsin is a rose-colored carotenoid-protein. In aqueous solution its absorption spectrum consists of a single broad band maximal at about 500 mμ. In light it bleaches in a succession of photochemical and thermal reactions to orange or yellow products, liberating in the process the carotenoid *retinene*$_1$. The latter substance has never been found in other tissues than the retina. In chloroform its spectrum consists of a single band maximal at about 387 mμ. Mixed with antimony chloride reagent it yields the deep blue color characteristic of a number of carotenoids in this test, due in this instance to a specific absorption maximum at 664 mμ. In the retina the mixture of retinene$_1$ and protein reverts to rhodopsin; in addition retinene$_1$ is converted to vitamin A_1. This possesses maximal absorption in chloroform at 328 mμ and with antimony chloride yields an absorption band at 615–620 mμ (crude extracts). In the intact eye vitamin A_1 also re-unites with protein to form rhodopsin. The rhodopsin system as a whole therefore constitutes a cycle of the form (78, 79, 81):


Rhodopsin
(500 mμ)
light
Vitamin A₁ + protein
Retinene₁ + protein
(328 mμ in CHCl₃)
(SbCl₃ → 615–620 mμ)
(387 mμ in CHCl₃)
(SbCl₃ → 664 mμ)

Porphyropsin likewise is a carotenoid-protein, distinctly purple in color. Its spectrum resembles that of rhodopsin in form but is displaced toward the red so that its maximum lies at about 522 mμ. Porphyropsin participates in a retinal cycle identical in arrangement with that of rhodopsin, but with new carotenoids in the positions of retinene$_1$ and vitamin A_1. The bleaching of porphyropsin liberates a substance analogous in its properties and behavior to retinene$_1$ called tentatively retinene$_2$. This possesses an absorption band in chloroform maximal at about 405 mμ, and yields with antimony chloride a band at about 705 mμ. In the retina it is converted simultaneously to porphyropsin and to vitamin A_2. The latter possesses a maximum in chloroform at about 355 mμ, and with antimony chloride yields a band at 696 mμ. The porphyropsin cycle therefore possesses the structure (83):


Porphyropsin
(522 mμ)
light
Vitamin A₂ + protein
Retinene₂ + protein
(355 mμ in CHCl₃)
(SbCl₃ → 696 mμ)
(405 mμ in CHCl₃)
(SbCl₃ → 705 mμ)

Spectra of the main components of the rhodopsin and porphyropsin cycles are shown in Fig. 4, those of the antimony chloride reactions with the retinenes and vitamins A in Fig. 5 and 6.

The behavior of both these systems is identical in all particulars, yet the spectrum of each component of the porphyropsin cycle is displaced 20–30 mμ toward the red from its analogue in the rhodopsin system (Fig. 4). It is known that displacements of this kind and degree accompany the addition to a polyene chain of one conjugated double bond (42). A small

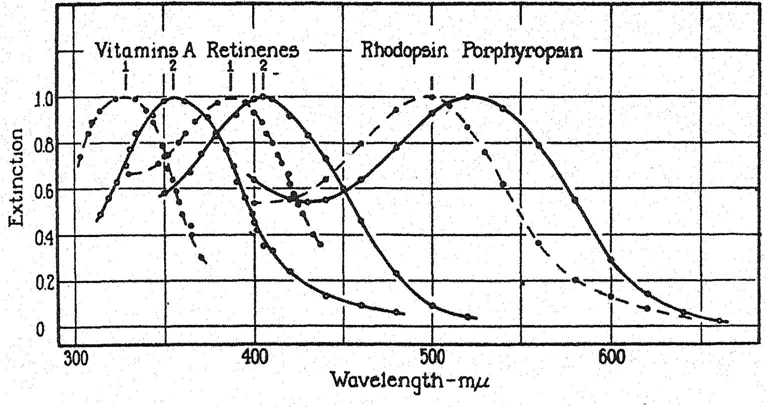


Fig. 4. The rhodopsin and porphyropsin systems
Spectra of crude preparations from retinas of the freshwater calico bass (solid lines) and the marine scup (broken lines). Rhodopsin and porphyropsin are dissolved in 1 per cent aqueous digitonin, the retinenes and vitamins A in chloroform. All maxima have been brought to the same height to facilitate comparison. (From the *Journal of General Physiology* (83)).

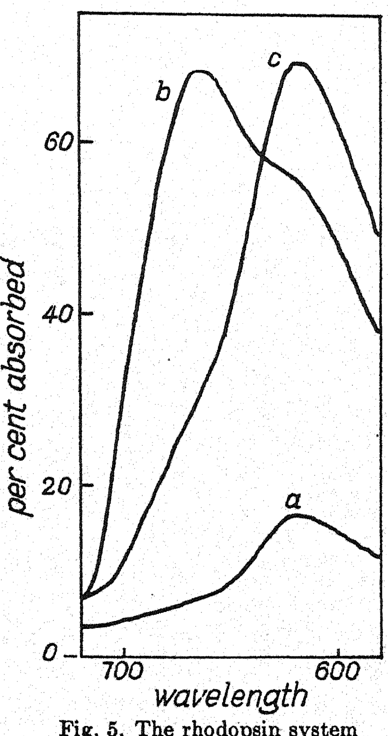


Fig. 5. The rhodopsin system
Spectra of the antimony chloride reaction with benzine extracts of retinas of the smooth dogfish. (a) Dark adapted retinas contain a small amount of the 618 mμ-chromogen, vitamin A_1. (b) Immediately following irradiation these same tissues yield a large quantity of the 664 mμ-chromogen, retinene$_1$, liberated in the bleaching of rhodopsin. (c) In retinas allowed to remain in the light at room temperature for about one hour retinene$_1$ has been converted to vitamin A_1. (From the *Journal of General Physiology* (83)).

structural change of this type in their carotenoid components is probably the sole difference between the rhodopsin and porphyropsin systems.

Gillam, *et al.* (27) presented what at the time appeared to be convincing evidence that vitamin A_2 is the next higher homologue of A_1, possessing one added ethylenic group (—CH═CH—) and the formula $C_{22}H_{32}O$. This possibility has since declined with the demonstration that the range of temperatures in which vitamin A_2 distills in high vacua is raised much less over that of vitamin A_1 than the possession of two added carbon atoms demands (29). Recently Karrer, *et al.* (35) have made the extremely interesting suggestion that vitamin A_2 is related to lycopene in the same manner that vitamin A_1 is to β-carotene, *i.e.*, that it possesses the structure of a dehydrogenated phytol. Evidence adequate to establish this formulation has not yet been presented.

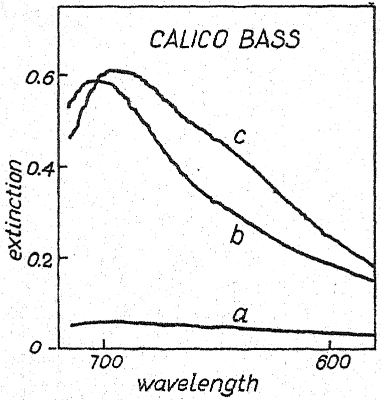


FIG. 6. The porphyropsin system

Spectra of antimony chloride reactions with benzine extracts of retinas of the freshwater calico bass. (a) Dark adapted retinas yield a trace of the 696 mμ-chromogen, vitamin A_2. (b) Immediately following irradiation the same tissues yield a large quantity of the 705 mμ-chromogen, retinene$_2$. (c) In retinas left in the light for about one hour the retinene$_2$ has been converted to vitamin A_2. (From the *Journal of General Physiology* (83)).

With this information as background one may proceed to examine the nature and distribution of retinal photoreceptor systems. Their study in invertebrates is barely begun. For a period the conviction had gained ground that invertebrates lack the vitamins A. These substances had not been identified in any invertebrate tissue. Gross hauls of marine zooplankton, composed primarily of invertebrate material, had yielded extremely little vitamin A or none at all (17, 26). It had been reported also that neither cockroaches nor clothes moths appear to require vitamin A in their diets (15, 53).

It is now known that the retinas of invertebrates contain high concentrations of vitamins A. Unlike vertebrates, however, in which large quantities of these substances are stored in the liver and other tissues, in invertebrates they are frequently and perhaps generally confined to the eye. For this reason the absolute quantities of these substances found in whole bodies and probably also the nutritional requirements of invertebrates are extremely low. The pre-eminently *visual* rôle of the vitamins A appears with great force in these animals; and indeed no evidence exists that in invertebrates they exercise other functions.

Among molluscs only the retina of the squid, *Loligo pealii,* has been examined (84). It contains 1–2 μg. of vitamin A_1 and about three times this quantity—measured as relative density in the antimony chloride reaction—of retinene$_1$. Most of the latter is bound in a light-stable complex which probably represents a retinal reserve. No trace of these or any other carotenoids was found in other squid tissues, or in extracts of whole bodies less the retinas. The quantity of vitamin A_1 remains constant in all states of exposure to light and darkness, and no evidence could be obtained that it participates at all in the visual processes. The irradiation of dark adapted retinas however liberates a considerable quantity of retinene$_1$. The photoreceptor system in this animal seems to consist of the simple cycle

$$\text{photopigment} \xrightleftharpoons{\text{light}} \text{retinene}_1 + \ldots.$$

Attempts to extract the photopigment into aqueous solution have not yet succeeded; though judging from measurements of the spectral sensitivity of the squid retina it possesses absorption properties resembling those of rhodopsin (Hartline, personal communication). Retinene$_1$ is not alone the predominant but also the active carotenoid in this tissue; it is in fact the vitamin A of the squid.

No arthropod systems have yet been examined as completely as this. For the most part experiments have been confined to the fractionation and identification of the carotenoids of the eye. In a number of marine crustacea—the green and fiddler crabs and the lobster—the eyes contain very high concentrations of vitamin A_1 in addition to astaxanthin and other carotenoids found throughout the integument. No trace of retinene$_1$ has as yet been found in these eyes (84). In a freshwater crustacean, the crayfish *Cambarus virilis,* the eyes contain both vitamin A_1 and retinene$_1$ in addition to astaxanthin.

The eyes of all the invertebrates examined therefore contain vitamin A_1; certain of them also retinene$_1$, in relatively high concentration. These substances reappear in the vertebrate retina as components of the rhodopsin system. In addition astaxanthin and smaller amounts of other carotenoids occur in the eyes of crustacea; but unlike the vitamins A they are widely distributed also throughout the integument of these animals, and appear to play no active rôle in vision.

The vertebrates introduce a new element in the distribution of visual systems, a basic cleavage in their composition between those forms which populate fresh water and those found in the sea. The retinas of all freshwater fishes so far examined possess the porphyropsin-vitamin A_2 system alone; and this is usually, though not always, accompanied by a great predominance of vitamin A_2 in the liver oils.

Marine fishes present the opposed pattern. All of them so far examined, teleost and elasmobranch, with the single exception of the Labridae, possess the rhodopsin-vitamin A_1 cycle alone. All without exception contain a great predominance of vitamin A_1 in their livers and other tissues. The labrid fishes—cunner and tautog—though permanently marine possess predominantly the porphyropsin system (82, 83).

A large number of fishes do not fit comfortably within this simple classification since they are capable of both marine and freshwater existence. Such euryhaline forms all seem to be restricted sharply in spawning environment, and on this basis may be divided into two groups: anadromous fishes, like the salmons, which spawn in fresh water; and catadromous fishes like the "freshwater" eel, which spawn in the sea. It is important to note that the spawning environment can serve as the permanent habitat of all these fishes. Migration is only a potentiality which they exploit in varying degree. The eel is really a marine fish with the peculiar capacity to enter fresh water, the salmon a freshwater fish capable of going to sea.

Of the anadromous fishes which have been examined, three genera of salmon—the brook and rainbow trouts and the king salmon—all possess mixtures of the rhodopsin and porphyropsin systems, and of vitamins A_1 and A_2, primarily the latter. The catadromous "freshwater" eel and killifish also contain mixtures of both systems, but in reverse proportion. Retinas of the anadromous white perch and alewife contain the porphyropsin system alone. All these animals therefore possess predominantly or exclusively the visual system and retinal vitamin A commonly associated with the environment in which they spawn (82, 85) (Fig. 7).

These relations are genetic, and at least to a first approximation independent of the immediate environment. The appearance of the porphyropsin system in the retinas of marine Labridae is one evidence of this. Salmons which had spent all their lives in fresh water still possess a high proportion of the marine-type rhodopsin system. Eels taken from landlocked freshwater ponds contain retinal and liver vitamin A patterns almost the direct reverse of those found in their permanently freshwater neighbors. Conversely alewives just entering fresh water on their spawning migration after years at sea contain almost exclusively vitamin A_2 in both retina and liver, like permanently freshwater fishes. It is clear that the choice between vitamins A_1 and A_2 as basis for the visual receptor system is a racial property, fixed more or less permanently in these animals, and associated in a genetic way with their environmental *origins* rather than with their immediate habitat.

How far backward does this peculiar cleavage in visual systems extend? It will be recalled that the only freshwater arthropod so far examined, the crayfish, contains vitamin A_1 just as do marine crustacea and the squid. There is as yet no indication that vitamin A_2 extends below the vertebrates.

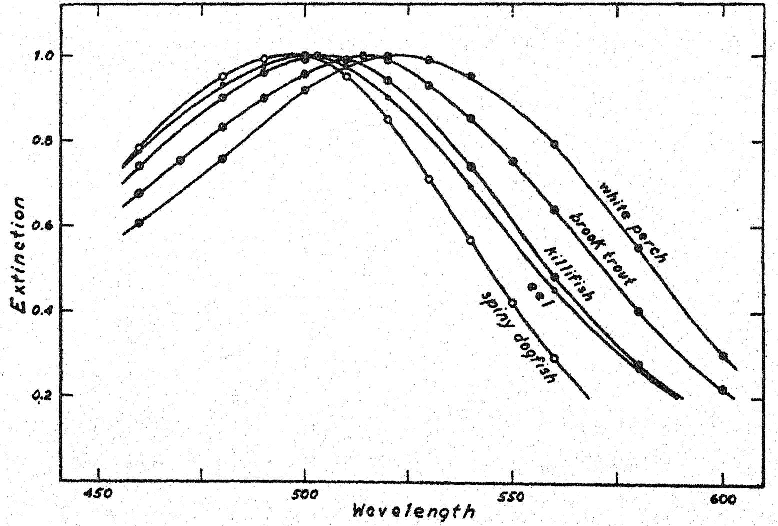


Fig. 7. Spectra of photosensitive pigments from fish retinas, illustrating the transition from an exclusively rhodopsin to an exclusively porphyropsin system

The permanently marine dogfish possesses rhodopsin alone, the catadromous eel and killifish predominantly rhodopsin, the anadromous brook trout primarily porphyropsin, and the anadromous white perch porphyropsin alone. (From the *Journal of General Physiology* (85)).

It is generally agreed at present that vertebrates originated in fresh water, and conceivable therefore that ancestral vertebrates already possessed the porphyropsin system. This is of course a matter not open to direct investigation. Probably the closest approach toward it however is offered by the cyclostomes, the most primitive of living vertebrates, all of which, like the presumed ancestral vertebrates, spawn in fresh water. The member of this group which has been examined, the sea lamprey *Petromyzon marinus*, is anadromous. Animals taken as they entered fresh water on their spawning run from the sea were found to possess an enormous preponderance of the porphyropsin system, like an anadromous fish (86).

Retinas of the African lungfish *Protopterus* have also been examined (unpublished observations). This animal is believed to have descended from a line of continuous freshwater ancestry, widely separated from that of modern freshwater teleosts, and close to that of the amphibia. The available data show that it too possesses the porphyropsin system (87).

The retinal use of vitamin A_2 therefore appears to be universal in freshwater vertebrates and to extend as far backward toward vertebrate origins as it is possible to penetrate. It probably is as ancient as the vertebrate stock itself. This contribution to the biology of the carotenoids, however, is curiously labile. Fishes during their evolution appear to have migrated repeatedly between fresh water and the sea, and appear as regularly to have oscillated between the fixed limits of the porphyropsin and rhodopsin systems. The euryhaline fishes represent intermediate types in this regard, both in their environmental habits and in the composition of their visual systems.

Vertebrate evolution has followed two pathways out of fresh water, one into the sea, the other to land. Both developments have led it back to the visual use of vitamin A_1; for only the rhodopsin system has as yet been found in permanently terrestrial vertebrates.

Interpolated between fresh-water fishes and land vertebrates are the amphibia. The position of these animals and the arrangement of their life cycles are closely comparable with those of euryhaline fishes. Most amphibia, like anadromous fishes, are hatched and spend a larval period in fresh water. Both groups thereafter ordinarily migrate for a period of adult growth, anadromous fishes to the sea, amphibia to land. Both groups eventually return to fresh water to spawn.

The common newt, *Triturus viridescens*, possesses such an "anadromous" history. Its retina contains the porphyropsin system alone, like that of many anadromous fishes. This observation marks the first appearance of vitamin A_2 within the amphibia (87). On the other hand, the frog, which has a similarly "anadromous" life cycle, is the classic rhodopsin animal, and most of what is known of the rhodopsin system has been learned from it. The distribution of visual systems within the amphibia, therefore, is still obscure; but it is clear that both rhodopsin and porphyropsin appear within this class of vertebrates.

No direct information concerning the visual utilization of vitamins A in reptiles is yet available. In the retinas of certain turtles, however, brightly colored oil globules are located in the position of filters between the inner and photosensitive outer segments of the cones. These are of three colors: red, golden-orange, and greenish yellow; and the notion has been prevalent for many years that they might constitute an apparatus for color differentiation comparable with three-filter arrangements used in color photography. The filter pigments of the turtle *Clemmys* have been examined. All of them are carotenoids, divisible roughly into astaxanthin, xanthophyll, and hydrocarbon (carotene) fractions, the colors of which simulate closely those of the retinal globules (90).

Among birds, the owl (41) and chicken (80) have been reported to possess rhodopsin. Beyond this information nothing is known of the retinal use of vitamins A within the entire class. The cones of a number of birds—for example, the chicken and pigeon—contain arrangements of colored oil globules similar to those described above in turtles. The filter pigments of the chicken retina have been isolated, and identified as astaxanthin, a mixture of xanthophylls resembling that in egg yolk, and an unidentified carotene resembling sarcinene, the pigment of the bacterium *Sarcina lutea* (90, 46). It can be shown clearly in this instance that astaxanthin is synthesized by the chicken, since it is absent from the yolk yet appears in the embryonic retina before hatching. No astaxanthin has been found in chicken tissues other than the retina.

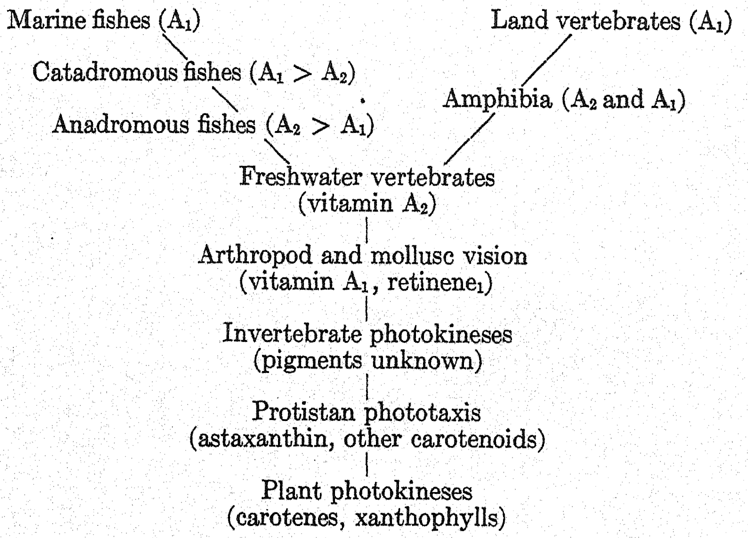
Among mammalian retinas, those of rats, rabbits, cattle, sheep, and pigs have been found to contain the rhodopsin-vitamin A_1 system alone (77, 81). Evidence also exists that the human retina (40) and those of the monkey, cat, and dog (41) possess rhodopsin. Throughout this entire class of vertebrates there is as yet no evidence that vitamin A_2 plays any part in visual processes.

The chemistry of cone vision is still obscure. Direct evidence has appeared for the existence of a special photopigment, iodopsin, in the cones of the chicken retina (80). Its similarity in chemical behavior to rhodopsin and porphyropsin suggests that it also may be a carotenoid-protein; and recent demonstrations that in human vitamin A deficiency the cones are affected in the same manner as the rods suggest that it may be dependent chemically upon vitamin A_1 (89, 33). In the absence of direct information, however, this is still very uncertain.

VII. Summary and Conclusions

In this paper an attempt has been made to gather together and evaluate the evidence that in plants as in animals carotenoids play a major rôle in the primary processes of photoreception. There is now good reason to believe that the light which stimulates photokinetic responses in a considerable variety of plant structures is absorbed by their carotenes and xanthophylls. In certain protista, and apparently concentrated in their eye-spots, these pigments reappear in company with a new and predominant carotenoid, astaxanthin, which has been found otherwise only in animal tissues. The chemical basis of photoreception in lower invertebrates is still unexplored. With the appearance of image-forming eyes in arthropods and molluscs the active rôle in photoreception appears to have passed in general to vitamin A_1 and retinene$_1$. With the freshwater vertebrates one encounters the porphyropsin system of the rods, based upon vitamin A_2. This thereafter remains closely associated with freshwater existence. From primitive stocks which probably possessed this system both marine fishes and terrestrial vertebrates were derived, both characterized by the return to the use of vitamin A_1 as basis for the rhodopsin system of the rods.

Interpolated between freshwater and permanently marine fishes are euryhaline forms like the salmon and eel. These possess both the rhodopsin and porphyropsin systems, frequently in mixtures, but always predominantly the system commonly associated with the environment in which the fish spawns. Similarly interpolated between freshwater fishes and permanently terrestrial vertebrates are the amphibia, in which again both systems appear, though the factors which influence their distribution are still obscure. These relations are summarized in the following diagram:


Marine fishes (A_1)
Land vertebrates (A_1)
Catadromous fishes ($A_1 > A_2$)
Amphibia (A_2 and A_1)
Anadromous fishes ($A_2 > A_1$)
Freshwater vertebrates
(vitamin A_2)
Arthropod and mollusc vision
(vitamin A_1, retinene$_1$)
Invertebrate photokineses
(pigments unknown)
Protistan phototaxis
(astaxanthin, other carotenoids)
Plant photokineses
(carotenes, xanthophylls)

It may be no more than an interesting coincidence that astaxanthin makes recurrent appearances in organs of photoreception. At present it seems to be the major pigment of the protistan eye-spot. It is found again in the eyes, as well as throughout the integument, of many crustacea. In the cones of certain birds and turtles it reappears as a filter pigment believed to play a rôle in color differentiation; and in the chicken it apparently is restricted to the retina.

Incomplete and frequently indecisive as these data are, they convey the strong impression that photoreceptor systems throughout the range of living organisms depend very generally upon carotenoids. These substances are drawn upon repeatedly, not only as active agents in photoreception but for accessory functions, as in sauropsidan cones.

In plants carotenoids fulfill functions other than photoreception, but none as yet known that is as general in scope or in which these pigments have so prominent a part. In warm-blooded vertebrates the symptoms of vitamin A deficiency indicate that this factor exercises widespread functions, reflected in the growth and general well-being of the organism. In cold-blooded vertebrates however only the retinal function of vitamins A is established; that it has further activities is suggested only by the presence of large stores of vitamins A in other tissues. In invertebrates even this indication fails; in those cases which have been investigated vitamins A appear to be confined to the eye, and there is no present reason to assume that they have any but visual functions.

The retinal rôle of vitamins A therefore is not the digression which its isolated position in mammalian vitamin A metabolism seemed to imply. On the contrary it is the only general function of this group of vitamins now known. It offers the only available basis for assessing their general zoological position and the added possibility that this is continuous with the position of the carotenoids in plants.

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The Significance of the Vitamin Content of Tissues

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I. INTRODUCTION

In this chapter it is hoped that there may be presented in a short space a rather broad view of the problem of vitamin distribution, particularly in animal tissues, which will be suggestive of additional research. A thoughtful study of the data under consideration leads one to realize how manifold the problems related to vitamins are, and how many questions of a fundamental nature are as yet unanswered. One is led to appreciate also how fragmentary, inexact and speculative is our knowledge as to the fundamental rôles played by the different tissues and organs of the body.

It would appear to be not an overstatement to say that vitamin research has the capability of revolutionizing the science of physiology and enriching it in every department. Vitamins may be regarded, from the standpoint of physiology, simply as a miscellaneous and heterogeneous collection of indispensable tissue constituents which were discovered by nutritional investigation. It took nutritional investigation using guinea pigs and dogs to point the way toward two new constituents of rat tissues which are of outstanding physiological significance for the rat; namely, ascorbic acid and nicotinamide. Nutritional investigations of yeasts and bacteria have led to the recognition of other highly significant constituents of animal tissues: pantothenic acid, biotin, inositol, *p*-aminobenzoic acid and folic acid.

These substances are, in most cases at least, vitamins for various animals, but their significance in the internal physiology of the animals where they are found may be just as great whether they are indispensable food constituents or are manufactured within the animals' tissues.

II. GENERAL DISTRIBUTION IN THE TISSUES OF VARIOUS ORGANISMS

1. *Vitamins A, D, E, K*

The terms "vitamin A," "vitamin D," etc., as applied to the fat soluble vitamins under consideration, do not of course refer to single chemical substances but rather to a nutritional source of a supposed physiological entity. In general these terms will be used in the usual loose sense in this discussion, though admittedly there would be an advantage in many cases if individual substances could be discussed and referred to by suitable names.

Various substances possessing vitamin A activity, notably the carotenes, are found in higher plants. In general, fungi including yeasts have not been found to be sources of vitamin A, though β-carotene and other carotenoids have been isolated from colored yeasts and other fungi (1, 2). Vitamin A seems to be very generally present in the livers of mammals (22 species, including man), birds (36 species) and reptiles (2 species). The amount in the livers of guinea pigs was very low in comparison with other livers (100 I.U. per gram) (3). In earlier experiments Karrer, von Euler and Schöpp (4) had found the liver oils of the following animals to be devoid of vitamin A: Bengal tiger, male lion, young male lion, raccoon, seal, cormorant, salamander and one species of reptile.

The presence of vitamin A (A_1 and A_2) in the livers of fishes is too well known to require emphasis at this point, and no attempt will be made here to review the extensive studies on this subject.

An experiment of unusual importance from the standpoint of the present discussion is that of Bowers and McCay (5) who raised 2.5 kg. of cockroaches on a vitamin A-free diet. A large sample of oil from these insects (150 g.) was found by colorimetric and animal feeding tests to be devoid of vitamin A.

The conclusions to be drawn from the facts cited above are that vitamin A appears not to be essential to the life of colorless plants, fungi and yeasts, and that even as highly organized a member of the animal kingdom as an insect can live its entire life history without having this vitamin in its tissues. Vertebrates—mammals, birds, reptiles and fishes—probably all utilize vitamin A as an indispensable tissue constituent and store it to varying degrees in their livers and elsewhere.

There appears to be no evidence of the widespread occurrence of vitamin

A in combined form though Lovern, Edisbury and Morton indicate that in fish liver vitamin A is at least in part associated with protein (6). Whereas carotenoid proteins are present in the retinas of various species, simple extraction methods are generally sufficient to remove vitamin A from tissues (7, 8) and the amount recovered is not increased by alkaline digestion. These findings indicate either a lack of combination or at most a loose combination with proteins or other colloidal cell constituents.

Comparatively little is known regarding the distribution of vitamin D in the tissues of animals. The livers of fishes and particularly the oils derived from them have, of course, been investigated extensively as commercial sources of vitamin D. Livers of mammals also have been found to contain some vitamin D though they are relatively poor sources. The qualitative finding that vitamin D is formed and is present in the skin is of interest, but practically no additional information regarding the distribution of the vitamin in the body of mammals has been found. It appears that practical considerations have been important in connection with research upon vitamin D. The fact that the vitamin can be produced cheaply by irradiation of suitable materials, and also the existence of at most small amounts of the vitamin in animal tissues, has made the investigation of the content of tissues unattractive as well as difficult. Scientifically, of course, the distribution of vitamin D in tissues is interesting and studies in this field might throw light upon its functioning.

It is commonly recognized that ordinary foods contain very small amounts of vitamin D and included among these are green plants.

Fungi, including yeast, are in general rich sources of sterols which are capable of becoming "vitamin D" upon irradiation. Mellamby, Surie and Harrison (9) found ergot to be rich as an antirachitic agent, but mushrooms were inactive. Scheunert, Schieblich and Rescheke (10), however, found that mushrooms, even when grown in the dark (cellar), contained 0.21 unit of vitamin D per gram. Those grown in the light, however, contained about three times as much. There is some doubt whether vitamin D enters indispensably into the physiology of fungi which live their entire life history in the dark. Yeast becomes a rich source of vitamin D upon irradiation, but its antirachitic potency when kept entirely in the dark is at most, very low.

Vitamin E appears to be rather widely distributed in the tissues of plants and animals. Part of it is stored in depot fats and unlike other vitamins it appears not to be concentrated in the liver. Mason (11) has recently determined by animal assays the approximate relative amounts of vitamin E in rat tissues. The "mean fertility doses" of the various tissues were from 30–120 g., and the relative amounts of vitamin E in the tissues assayed are given in Table II. He states that the concentration of the vitamin in those tissues which undergo alteration after pathological deprivation (*e.g.* fetus, testes, musculature, uterus, brain and spinal cord) differs in no significant way from the other tissues. That the vitamin E content of different species varies through fairly wide limits is indicated by the experiment of Underbjerg (12) in which seven goats were fed a diet which was so deficient in vitamin E that it could not support reproduction in rats. In 4½ years the herd had multiplied to 48. However, the milk and flesh of the goats fed the vitamin E deficient diet would not cure rats which were sterile because of lack of vitamin E, whereas the flesh and milk of normal goats would cure such sterile rats. Goats either do not require vitamin E or more likely their requirement is much smaller than that of the rat. Evans (13) and others have discussed the functions of vitamin E aside from its relationship to reproduction, but not on the basis of the content of the tissues involved.

The alleged occurrence and functional significance of vitamin E in "royal jelly" (14) has not been confirmed by later investigations (15, 16). The relationship of vitamin E to insect life appears to be unknown.

"Vitamin K" is widely distributed in the biological kingdom, having been found in the livers of various animals, in numerous vegetable sources, in feces and in bacteria. It is evidently produced by certain bacteria, as in putrefied fish meal, and acts as a "growth substance" for certain others (17). It was found not to affect yeast growth or respiration (18). Not enough is known about the distribution of vitamin K to give much clue as to its significance. It is obvious from its functioning in bacterial metabolism that it may function otherwise than in blood coagulation.

2. Ascorbic Acid

The literature dealing with the distribution of vitamin C is voluminous and the wide distribution of ascorbic acid in the tissues of common mammals is well known. It has been determined by titration to be present in substantial amounts in 13 marine invertebrates and 7 marine plants (19), in earthworms, sea urchins, 6 species of mollusks and in crustaceans (20). The distribution of vitamin C in the organs of fishes and crabs (21) certainly indicates its functional importance in these organisms. Cockroaches fed a sterilized vitamin C-free diet remained prolific for years and contained 0.10 to 0.19 mg. of ascorbic acid per gram when assayed. Cockroaches at large had the same vitamin C content (22). If we can generalize from this experiment we may conclude that ascorbic acid is present in insect tissues. Using a silver nitrate staining technique, evidence has been presented for the occurrence of vitamin C in bacteria, molds, protozoa, lichens, algae, diatoms, yeasts and various fungi. King (23) says with regard to silver nitrate technique, "There remains, however, some question concerning the degree of interference by minute amounts of other constituents of the cell. If the silver deposit is a valid measure of ascorbic acid in all the cells studied, then one must conclude that such organisms as bacteria and yeast normally contain the vitamin." It is well recognized that yeast, for example, is practically devoid of antiscorbutic potency and no "vitamin C" content is claimed for the commercial product.

To the writer it appears that our knowledge with regard to the distribution of ascorbic acid in lower organisms is in an unsatisfactory condition. Evidence from the standpoint of actual possession of antiscorbutic potency or ability to yield ascorbic acid in isolation procedures has not been forthcoming so far as many organisms are concerned. One cannot be sure on the basis of present evidence that ascorbic acid functions in all types of lower organisms. No conclusive evidence has been presented to indicate its universal presence in living matter.

Holtz and Walter (24, 25) have presented evidence that vitamin C occurs in animal tissues partially in a combined form. Upon addition of protein precipitants to tissue extracts, a substantial part of the vitamin C is found in the precipitate from which it may be released by acid treatment. The combined form of ascorbic acid is said to be a reserve storage form of the vitamin and to be relatively resistant to irreversible oxidation. The presence of ascorbic acid in bound form in vegetables, for which potatoes were used as a convenient source, has been demonstrated by Reedman and McHenry (26).

3. B Vitamins

Long before "vitamin B" became resolved into a number of components, the very widespread occurrence of this indefinite agent was a notable fact. Various lower forms of life (bacteria, yeasts, molds, insects, etc.) were able to live without vitamin A or C, but whenever a vitamin was found to be required it was of the "vitamin B" type.

In recent years the exceedingly widespread occurrence of individual members of the B family has become more apparent on the basis of many hundreds of studies. By the use of microbiological tests it has been possible recently to gain more evidence on the question of the distribution of B vitamins (27). The B vitamins studied included thiamin, riboflavin, niacin, pantothen (28), pyridoxin, biotin, inositol and "folic acid." These were found uniformly to be present in numerous tissues (autolyzates) of rats, mice, cattle, hogs, and chickens, in embryonic rat and chick tissues, in mixed foods and in urine.

An extension of these studies has shown that each of the "B vitamins" is always found in every biological source tested. In Table I are given the results of a series of assays of materials derived from widely separated phyla in the biological kingdom. Aside from the samples listed, the tissues of higher plants and animals also show uniformly the presence of all of the eight vitamins considered. In Table I the amounts obtained are derived from extracts obtained by enzymatic release of the B vitamins, incubating the materials tested with "clarase" and papain (29).

TABLE I

B Vitamins in Lower Organisms

	Thiamin	Riboflavin	Nicotinic Acid	Pantothenic Acid	Pyridoxin	Biotin	Inositol	Folic Acid
Fish (*Cyprinidae*)	2.9	1.6	23.9	7.5	1.1	.095	274	1.64
Frog (*Rana*)	1.4	2.52	11.7	3.7	1.2	.126	268	.316
Snake (*Thamnophis*)	1.0	9.11	28	5.1	.80	.05	212	1.71
Chick embryo	.58	.93	28.4	26	.47	.123	83	2.49
Red ant (*Dolichoderus*)	3.2	6.08	20.5	12.5	.67	.160	964	1.54
Cockroach (*Periplaneta Americana*)	4.4	7.11	33	17.5	1.3	.13	363	.85
Termites (*Zootermposis*)	2.3	4.75	32	16	.32	.119	389	2.23
Dros. virilis larvae, N. Y.	4.2	8.11	36.5	20.0	1.3	.352	155	11.12
Dros. virilis larvae, N. O.	4.4	8.22	37.5	20.5	.94	.374	249	18.66
Oyster (*Mytilus*)	1.8	2.09	11.7	4.9	.45	.087	444	2.26
Earthworm (*Lumbricus terrestris*)	2.5	8.0	15	3.2	.29	.079	164	.706
Protozoa (*Tetrahymena geleii*)	5.0	2.22	11.7	13.8	3.1*	.098	432	3.04
A. aerogenes, aerobic	2.19	9.0	49.1	30	1.4	.80	279	†
S. marcescens	4.56	5.9	40.1	20.95	1.79	.699	277	2.75
P. fluorescens	5.46	14.07	44	19.1	1.19	1.49	357	1.84
C. butylicuminaer	2.69	15.95	73	26.9	1.79	.49	252	.812
Mushroom (*Coprinus atranentarius*)	1.1	3.26	68.5	17	.45	.18	168	.98
Brewer's yeast	8.5	15.2	126	42.5	1.0	.071	278	1.05
	.522	.671		.840	.005	.160	.219	−.160

* Assay not entirely satisfactory (other values obtained were even higher).

† Vitamin *not* absent; analysis incomplete.

An observation made in connection with the tissues of a given animal (30) appears to hold, though less generally, throughout the biological kingdom; namely, the existence of a correlation between the content of the various B vitamins. In other words, there is some tendency for the B vitamins to be associated together quantitatively. Sources which are rich for one B vitamin are likely to be rich for at least some other members of the family.

When correlation coefficients were determined between the contents of the other B vitamins with that of nicotinic acid in the diverse organisms in Table I, the following values were obtained: Thiamin .52, Riboflavin .67, Pantothenic acid .84, Pyridoxin .005, Biotin .160, Inositol .219, and Folic Acid −.160. The implications of some of these findings will be discussed in a later section.

In case of nicotinic acid (or amide), riboflavin and thiamin, enzyme studies have proved that combinations of these with other molecules exist and are functionally important, and experience in extracting the other "B vitamins" shows that these in general are tied up in tissues with colloidal constituents.

If liver from a freshly killed animal is extracted immediately with hot water it yields a very small amount of the total pantothenic acid which it contains. The rest may be freed by autolysis or incubating with enzymes (31). Acid or alkaline hydrolysis cannot be used because the pantothenic acid itself is readily split (32).

Biotin is rather firmly bound (presumably to proteins) in tissues. The fact that it is partially released by autolysis, and more completely by incubating with enzymes or by acid hydrolysis has been thoroughly demonstrated (33, 34). The first evidence as to the autolytic release of biotin (33) served as a clue to identify biotin with "vitamin H" (35). The evidence indicates that biotin exists in several combinations in nature. One of the most interesting is its combination with "avidin," the protein in egg-white which causes egg-white injury (36, 37, 38).

Inositol is known to be released from tissues by enzymatic action, and Woolley (39) has recently shown that during incubation of eggs the inositol originally present in combined form is largely freed.

Folic acid is likewise freed from tissues by autolysis and even more completely by incubating with enzymes. Conditions have been developed by which it is possible by one treatment with a mixture of "clarase" and papain to release fairly quantitatively all of the B vitamins considered, from their combinations (29).

III. Distribution of Vitamins in the Tissues of Mammals

1. *Tables and Discussion*

In Tables II and III is given information regarding the amounts of the different vitamins obtained from various rat tissues and from human tissues.

As will be discussed in more detail later, the value for the content of a given B vitamin in a particular tissue appears to be a great deal more constant than the corresponding value for the content of vitamin A or C. In 56 pairs of B vitamin assays of male and female rat tissues, there were three cases only in which one value was about double the other; in the 53 other cases the higher value was as a rule not more than 50 per cent greater than the other and often agreed within 10 or 20 per cent. It will be noted that in general the agreement between the individual human cases is good insofar as the content of the B vitamins in the tissues is concerned. In contrast to this Yavorsky, Almaden and King (40) found that in a series of vitamin C assays "individual cases varied from approximately 3 times higher than the average down to less than 0.1 the average values." In the data of Clausen and McCoord (41[1]) the vitamin A content of livers of the same species differed in two cases (cat and guinea pig) by over 100 fold.

TABLE II

Vitamin Content of Rat Tissues

	Vitamin A	Vitamin C	Vitamin E, Relative Values	Thiamin	Riboflavin	Nicotinic Acid	Pantothenic Acid	Pyridoxin	Biotin	Inositol	Folic Acid
Liver	7.6	230	1	7.6	27	178	112	1.9	.84	513	6.1
Spleen			4	2.7	3.8	69	9.0	.39	.0725	1,075	6.1
Heart	.2	120	3.4	7.3	12.4	123	35	1.4	.38	610	2.9
Kidney	2.7	140	2	4.2	27.5	116.5	34	1.6	.79	1,540	8.3
Muscle	.7	60	2	1.2	1.9	81	5.0	1.2	.051	170	1.26
Lung	3.3		3.4	2.4	4.3	51	9.0	.285	.078	1,020	3.7
Brain	0	360	—	4.4	3.1	64	11.5	.94	.10	1,650	3.1
Adrenals	49	2,940	—		18.5	125	36	(.25)	(.63)	(450)	3.7
Testes		310	2	6.9	3.0	25.2	20.0	0.31	.07	402	.85

Vitamin A values International Units per gram of moist tissue.

Vitamin E values are expressed in arbitrary (relative) units.

Folic Acid values are expressed in units per gram. 1 μg of "potency" 40,000 = 1 unit.

All other values are in terms of μg per gram moist tissue.

In considering content of B vitamins in different species, it appears that there is some tendency for the content to be lower in larger animals. However, this may be reversed in some instances in the mouse and rat. Beef tissues appear, when compared to the rat tissues, to be very rich in inositol, and the beef might with some justification be designated as a "high-inositol" animal. Pork is well recognized as an unusually rich source of thiamin,

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[1] The author wishes especially to express his thanks to Drs. S. W. Clausen and A. B. McCoord who allowed him access to information in advance of publication, with regard to their investigation of the vitamin A content of animal tissues. Part of this material was presented before the American Society of Biological Chemists in Boston in April, 1942.

TABLE III

Vitamin Content of Human Tissues

Normal Human Tissue	Vitamin A	Vitamin C	Thiamin		Riboflavin		Nicotinic Acid		Pantothenic Acid		Pyridoxin		Biotin		Inositol		Folic Acid	
			♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂
Heart	1.4	21.0	3.0	4.5	7.8	19	40.5	41	16	13	.91	.64	.17	.19	450	516	.9	1.1
Adrenal	6.0	230	1.6	1.5	8.2	11.3	30.7	24.4	8	6.1	.19	.14	.35	.23	250	688	1.1	1.1
Cerebral tissue	.5	110	1.4	1.8	2.1	2.8	19.8	19	13	16	.6	.7	.03	.08	1,582	1,420	.7	1.5
Liver	156	64	2.0	2.5	15.9	15.9	53.5	67	31	45	1.9	3.0	.62	.77	833	680	7.6	11.4
Lung	1.2	45	1.5	.6	1.6	1.9	18.4	18	5	2.8	.24	.07	.02	.01	397	350	1.3	1.2
Kidney	2.7	47	3.6	2.4	24.7	19.8	33.3	49	16	19	.73	1.4	.58	.67	861	1,242	1.8	2.3
Ovary			.61		4.3		18.3		4		.15		.03		581		1.1	
Testes				.55		1.7		8.0		2.9		.09		.05		667		8
Mammary gland			.43		2.4		10.2		3.9		.43		.04		270		.44	
Stomach	1.3		1.0	.56	5.2	5.3	30.3	18.8	6.5	5.6	.38	.18	.19	.11	469	1,236	1.0	1.5
Colon	.8		1.0	1.3	2.1	2.4	24	13	10.5	3.9	.32	.21	.08	.09	478	779	1.6	2.0
Ileum	1.3		1.1	.4	2.9	5.0	29	19	5.3	3.6	.34	.17	.06	.06	406	867	1.2	1.6
Seminal duct				.69		1.0		9.2		1.9		.04						.87
Skin	1.3			.52		1.5		8.2		2.7		.08		.01				1.0
Spleen	1.0	81	1.1	1.3	3.0	7.2	30.4	22	5.4	4.8	.12	.06	.04	.06	346	1,110	2.6	3.4
Skeletal muscle	.5		.84	1.3	2.0	2.9	50	48	10	18	1.1	.72	.02	.04	453	197	.7	.8
Smooth muscle			1.2		2.3		31		6.2		.53		.06		577		.97	
Rectum					1.6		26		4.6		.11		.04				1.5	

but in this case the situation is different because whereas most of the beef tissues are relatively rich in inositol, only hog *muscle* is rich in thiamin. The other hog tissues in general have less thiamin than the corresponding beef tissues.

An observation which is suggestive is noted by Bessey and King (42), "The vitamin (C) content of the rat, rabbit and chicken tissues where the vitamin is not a direct dietary factor but under physiological control, is higher than that found for guinea pigs receiving an apparently generous vitamin supply." A parallel case may be that of nicotinic acid which is synthesized in the rat and is therefore under physiological control. When calculated on a calorie basis the nicotinic acid content of the rat carcass appears to be high (43). No comparable assays of tissues of animals that require nicotinic acid are available.

A highly interesting and apparently perfectly definite observation with regard to ascorbic acid is that the content of tissues decreases steadily with age even when the animals are kept on the same diet (42, 44). Variations of the content of B vitamins with age are not great as a rule. They have not been extensively studied (45).

2. Correlation with Respiratory Activity of Tissues

Rather loose statements are often made with regard to how the vitamin contents of tissues may vary with the respiratory activity of the tissues.

In order to place some of the available information on as exact a basis as possible, nine rat tissues were selected, for which at least the oxygen consumption rate had been measured repeatedly and apparently with reproducible results. These tissues (which are the same as those listed in Table II) are listed in Table IV with the oxygen consumption data and also in most cases the anerobic glycolysis data.

The values for the vitamin A, ascorbic acid, vitamin E, thiamin, riboflavin, etc., content of each of the tissues (Table II) were correlated mathematically with each of the corresponding values in both columns of Table IV. The corresponding correlation coefficients were obtained (Table V).

When in the ascorbic acid calculations the adrenal gland was excluded from consideration (it has a very high content of ascorbic acid, which may be stored and hence non-functional in the gland) the correlation coefficients became (second line Table V) .198 and .809 respectively.

Some of the correlation coefficients are of such magnitude that they may be considered significant: Riboflavin content has a significant positive correlation with aerobic respiration and a negative correlation with anerobic glycolysis. Biotin and inositol have positive correlations both with aerobic respiratory rate and anerobic glycolysis rate. Thiamin, niacin, pantothen, pyridoxin and folic acid do not show significant correlations with either aerobic or anerobic activity.

TABLE IV

Aerobic Respiration Rates and Anerobic Glycolysis Rates of Rat Tissues

	$Q_{O_2}^{(-)}$	Q_G^N
Liver	11.6	3.3
Spleen	11.8	8.3
Heart	6.7	—
Kidney	21	3.7
Muscle	5.4	—
Lung	7.8	1
Brain	10.7	19.1
Adrenals	10	4
Testes	12.3	7.2

TABLE V

Correlation of Vitamin Content with Oxygen Consumption and Anerobic Glycolysis (Rat Tissues)

Vitamin	Correlation Coefficient	
	$Q_{O_2}^{(-)}$	Q_G^N
Vitamin A	.098	−.242
Ascorbic acid	−.075	−.235
Vitamin E	−.277	.140
Thiamin	.168	−.018
Riboflavin	.594	−.480
Niacin	.107	−.337
Pantothen	.205	−.344
Pyridoxin	.227	−.037
Biotin	.577	.573
Inositol	.572	.470
Folic acid	.223	−.303

IV. Significance of Distribution with Regard to the Functioning of the Respective Vitamins

1. *Vitamin A*

The distribution of vitamin A is perhaps the most erratic of any of the known vitamins and to obtain any clues with regard to its functions from known facts regarding its distribution would be very difficult. For example, while vitamin A is usually found abundantly in the livers of animals, there are apparently examples, cited above, where it is missing. While it is generally more abundant in the liver than elsewhere, certain intestinal fats of fishes contain 60–70 per cent vitamin A or its esters (46). In dogs the depot fats may contain as much or more vitamin A than the liver (41).

Recently Popper (47) has used fluorescence microscropy in studying the vitamin A content of human tissues. The same method has been applied to animal tissues and promises to be a valuable research tool, particularly with respect to localizing the vitamin A within tissues (48).

The importance of vitamin A in vision is often emphasized, but one would not guess its importance on the basis of its distribution in mammals. In the body of a dog, for example, there may be a total of 300 mg. of vitamin A and yet the amount in the eyes is only about 4 μg, or approximately .001 per cent of the total (41). However, when an animal is severely depleted, vitamin A remains in the eye (if fluorescence microscopy is a reliable guide) after it has disappeared from other tissues (48). The retinas of different species vary greatly in the amounts of vitamin A which they yield. In some specimens of gold fish, there is said to be nearly as much vitamin A in the retina as in the rest of the body (46). The total amounts of vitamin A present in the tissues of different animals in which it is functional varies through rather wide limits. Calculations derived from the data of Clausen and McCoord (41) have shown that the whole carcasses of the animals studied had the vitamin A contents indicated in Table VI.

TABLE VI

Vitamin A Contents of Different Species

	International Units per kg. of Carcass			Per cent in Liver
Dog	7,925	21,000	27,500	19, 16, 43
Cat	2,100	56,400		24, 92
Rat	24,000			97
Gopher	11,800			67
Rabbit	1,840	2,500		35, 21
Guinea pig	29,500	2,040		92, 10
Monkey	6,150			67
Chicken	4,400			50
Human	5,500	4,680		69, 85

The apparent absence of vitamin A from certain livers has been mentioned. In the study of Clausen and McCoord (41) the following tissues were found to be devoid of vitamin A in at least one specimen: lung (dog, guinea pig); small intestine (rabbit); large intestine (cat); stomach (guinea pig, cat); heart (guinea pig); spleen (rabbit, guinea pig); pancreas (rabbit); ovaries (rabbit); thyroid (rabbit, both specimens); adrenals (gopher).

One is tempted to conclude from all these data that vitamin A is not essential for cellular metabolism in mammals, and that its occurrence in body fat is in part responsible for its wide distribution in the body. Its function in connection with vision would appear to be only one of its functions. Its importance for epithelial tissue has long been recognized, and its *apparent* absence (in some cases) from tissues of this character may be due to their ability to obtain vitamin A from adjacent fatty tissue.

It must be admitted that the general functions of this vitamin, which is of early enough vintage to have been designated by the initial letter of the alphabet, remains at present very much obscured, and the present knowledge regarding its distribution does not do away with the obscurity.

2. Ascorbic Acid

This vitamin has been available in pure form for a number of years and methods for its quantitative determination have been used extensively. Except for the lack of accurate knowledge with regard to lower organisms, information regarding the distribution of ascorbic acid is very abundant.

Giroud (49) has reviewed the subject of its distribution in organisms (particularly animal tissues) and has discussed the significance of the findings, and it is not possible to review here the voluminous literature.

The study of the distribution of ascorbic acid has contributed relatively little to our knowledge of the functions of ascorbic acid. King (50) refers with good reason to "the baffling uncertainty regarding its apparent functions." It is quite reasonable, however, that a knowledge of its distribution will ultimately help in determining what its functions are.

The clear-cut facts with regard to the effects of vitamin deficiency and plethora on the formation of collagenous intercellular material in bones and teeth as well as other tissues (51) remains enigmatical so far as the mechanism is concerned, and there is nothing about the vitamin distribution which would suggest this function.

The fact that diphtheria toxin causes losses of ascorbic acid from the adrenals, pancreas and kidneys while it is being detoxified is highly significant (52). It would appear that the adrenals have a detoxifying function and the fact that the loss was greatest from this gland is not entirely out of line with the suggestion made earlier in this chapter that ascorbic acid may be stored in the adrenal gland.

The oxidation-reduction function of ascorbic acid, which has not received very substantial support so far as animal tissues are concerned seems not to be out of line with the observation (p. 245) that when the adrenal gland is left out of consideration, there is a high positive correlation between the ascorbic acid content of rat tissues and their anerobic glycolysis rates.

The presence of ascorbic acid in relatively high concentrations in the pituitary gland (pars intermedia) and the adrenal glands of various animals is a striking fact. There is apparently no known connection between ascorbic acid and the hormones produced by these tissues, nor is there any apparent reason why these two glandular tissues (which have no known relation to each other) should be extraordinarily rich in this vitamin. These facts accentuate the "baffling uncertainty" with regard to the functions of ascorbic acid.

It seems probable that ascorbic acid has functions other than those relating to the formation of collagenous material or counteracting bacterial toxins, because it seems to function in plant as well as animal physiology. It may be concerned with oxidation-reduction systems in plants as well as having other functions.

3. B Vitamins

a. Individual Members. The fact that hog tissues, except for muscle, are relatively poor in thiamin (p. 238) suggests the possibility that pork muscle contains some agent capable of binding thiamin to an unusual degree, and that other tissues maintain a relatively low level because of competition with this supposed agent.

The sex differences which appear in the distribution of thiamin between the adrenal cortex and medulla, in the bull and cow (53) are interesting because of the possible relation to the hormones from these glands.

The finding that raw fish, as fed, contains something which inactivates thiamin (54) opens interesting questions with regard to whether this substance functions in fish physiology and how, if it is present in living fish tissues, the fish are prevented from becoming deficient since they evidently contain an excess of the inactivating substance. Fish (cooked) are, in general, a reasonably good source of thiamin and presumably thiamin is just as indispensable to cellular activity in fish as elsewhere in the biological kingdom. Possibly the inactivating substance is localized and acts in a regulatory fashion.

In the developing egg, avidin, which has the ability to combine with and inactivate biotin, gradually disappears during incubation. Presumably it acts in a regulatory fashion since preliminary experiments by Taylor (55) indicate that the biotin level in the developing embryo is important.

Over half the nicotinic acid in the livers of rats and dogs is found to be in some other form than the usual nucleotides. Twenty per cent of the muscle nicotinic acid of dogs (but none in rats) is in non-nucleotide form. In neither animal is there any nicotinic acid in the kidney cortex other than that present as coenzymes 1 and 2 (56, 57). These facts indicate that nicotinic acid has functions other than those commonly ascribed to it.

The adrenal necrosis in rats caused by a deficiency of pantothenic acid, which has recently been confirmed again (58), is in line with the finding that this gland is, aside from the liver, perhaps the richest source of pantothenic acid in the body of the rat. There are indications that a part of the liver pantothenic acid is stored, in which case the adrenal gland may have the highest concentration of the functional vitamin.

The finding that the vitamin B_6 in animal tissues is to a considerable extent in a form which is not pyridoxin (59) complicates the question of distribution. Pyridoxin and "pseudopyridoxin," as the new substance is tentatively called, are alike in their effect upon yeast growth and are determined together in the microbiological method of Williams et al (60). "Pseudopyridoxin" is very much more effective as a nutrilite for lactic acid bacteria and is differentiated from pyridoxin because of this fact. Additional information regarding the exact distribution of pyridoxin and "pseudopyridoxin" must await further study.

Inositol is the one member of the B vitamin family which occurs in largest absolute amounts. Its distribution is erratic when different organs are considered or when different organisms are compared. Beef heart (autolyzate) is about 52 times as rich as rat muscle. Other B vitamins do not show such wide ranges of concentration as this. Plant tissues are often very rich as compared with animal tissues.

The distribution of "folic acid" (61) and its enzymatic release from tissues gives a substantial basis to the opinion that it is of physiological importance for animals. Its properties as a nutrilite for microörganisms is striking, and even though its vitamin properties for animals have not been adequately demonstrated, its importance in physiology seems assured.

b. General. One of the striking observations with regard to the distribution of B vitamins is that generally speaking there are no very rich or very poor biological sources of any member of the group. Even with the wide variation in organisms represented in Table I, the best sources of a given vitamin are seldom more than 10 or 15 times as rich as the poorest sources. The drosophila larvae are unusually rich sources of folic acid (about 60 times as rich as the poorest source), but if these are excluded the richest source is only ten times that of the poorest source. Even among the individual tissues of an organism there is not a wide spread of concentrations, as may be noted by inspection of Tables II and III.

If the tissues of a series of the same species of animals are assayed, the individual tissues of the same kind have contents which are remarkably concordant (p. 237). However, the order of richness of the various tissues is variable from animal to animal, that is, the organ or tissue which is richest (or poorest) in one species of animal will not necessarily be so in other species.

The relative constancy of the B vitamin content of tissues is in keeping with the observation made some years ago by Stare and Elvehjem (62), who found that the tissues of chicks and rats in various nutritional conditions did not vary greatly in their respiratory rates. If, as is commonly supposed, the B vitamins as a group are intimately concerned with cellular metabolism, the relative constancy of B vitamin content would be reflected by a relative constancy in respiratory rate. Of course severe deficiencies do result in lowered content of specific B vitamins as has been demonstrated a number of times.

The association of the various members of the B family of vitamins in the same sources has already been mentioned. This association suggests similarity of function. The question of partial replaceability of one B vitamin for another has not been settled definitely in all cases, though the general impression is that each vitamin is required to a certain extent, which is largely independent of the supply of the others.

The probability that the functions of the B vitamins are similar is enhanced by the experiments of Taylor (63), who found that placing hens on a diet fortified with pantothenic acid resulted in chicks with altered distribution of several of the B vitamins in the liver, brain and heart. Further experiments following this suggestive line of investigation would be valuable, since they might throw much light on the interrelations between the B vitamins.

The general lack of correlation between B vitamins and endocrine function (as well as a similar lack of correlation in the case of vitamin C) seems to be apparent. The distribution of B vitamins in the anterior and posterior portions of the pituitary gland (beef) was almost exactly the same in spite of the fact that these two portions of the gland are entirely different in endocrine function (64). It would seem that in this case contiguity rather than physiological similarity of tissue might have something to do with the close resemblance between the two portions of the gland.

It seems significant that of the insects tested (Table I), ants, cockroaches, termites, fruit fly, 2 strains of larvae, all were rather rich and relatively uniform in their content of thiamin, riboflavin, niacin and pantothenic acid, but the contents of the other B vitamins were highly variable. In the bacteria and the yeast, the same four vitamins tended to be abundant, the pyridoxin and biotin were somewhat high but irregular, while the inositol was low and the folic acid irregular. One might conclude from a study of these data that thiamin, riboflavin, niacin and pantothenic acid are peculiarly related one to another. This conclusion is supported by the high correlation coefficients (p. 235) between the contents of thiamin, riboflavin and pantothenic acid with those of niacin (Table I).

As study progresses it seems inevitable that the different members of the B family of vitamins will fall into groups within which particular similarities will be apparent. A study of the correlation coefficients given in

Table V may be worth while in this connection. It would be dangerous to place too much weight upon the correlation coefficients listed, but some of the values are suggestive. The relatively high positive correlation of riboflavin content with aerobic respiration and the negative correlation with anerobic glycolysis is in keeping with the position of riboflavin in biological oxidation schemes.

If the results in Table V have meaning they suggest a functional resemblance between biotin and inositol. Thiamin, niacin, pantothenic acid, pyridoxin and folic acid may be grouped together as showing no substantial correlation with either aerobic respiration or anerobic glycolysis, but this could not be taken to mean anything with regard to functional similarity or dissimilarity. The possibility of correlation of functions of pantothenic acid and riboflavin have been indicated in other work (65).

The fact that the ascorbic acid content correlates to a high degree with anerobic glycolysis when the adrenal glands are left out of consideration suggests the possibility that ascorbic acid may function in glycolysis and that the adrenal glands serve predominantly as a storehouse for this vitamin. Whether speculative predictions arising from data of this sort will be useful must be decided in the future.

V. GENERAL OBSERVATIONS

It is commonly assumed that vitamins act essentially as catalysts. It seems on the basis of known enzyme systems that if each of the known and unknown B vitamins were to be the prosthetic group of an enzyme there would be a plethora of enzymes in cells. Some students of the subject of cellular metabolism evidently incline toward the view that there are already almost enough recognized enzyme systems to perform the functions of the cell. Though this may be seriously doubted it seems that possibly some of the vitamins act not as catalysts in the stricter sense but rather as "promoters." Industrial catalysis could not function without the little-understood promoters which in small amounts tremendously affect the rates of catalytic reactions. The fact that the promoters and their action are not well understood by the physical chemist does not make them any less useful or indispensable. Possibly the same type of phenomena will some day be recognized in the field of biochemical catalysis, and will throw light on the functions of the B vitamins. Possibly the possession of multiple functions is based upon ability of some of the vitamins to act as promoters in several different reactions. Replaceability of the various B vitamins for each other, if it exists, would be suggestive in this connection.

A more complete knowledge of the distribution of the B vitamins, accompanied by additional knowledge regarding their functions, should lead to a more intimate understanding of what the various tissues and organs of the body do. The fact that the liver holds a relatively large quantity of most of the vitamins points either to the possibility that it possesses many functions or else that it is a storehouse or depository for vitamins. The latter is known to be true in some cases. Because liver is such a "catch-all" for vitamins the presence in liver of a substance possessing physiological activity of any sort may be taken as an indication of general physiological significance of the substance in question. The sooner the numerous physiological principles present in liver are identified the more rapidly will the vitamin picture approach completion. The efficacy of liver and extracts from it for the amelioration of anemias is probably due to a combination of such factors.

The distribution of B vitamins must have its bearing upon the problem of malignant growth. It appears from results obtained in the author's laboratory that cancer tissue derived from various sources in various ways has some of the characteristics of a special type of tissue, with its own characteristic distribution of B vitamins (66). Certainly on the basis of information now available, cancer tissue (using the term in an inclusive sense) shows certain regularities which it would be difficult to explain on any other basis. With added knowledge regarding the distribution and functions of the B vitamins will come at least more insight into the peculiarities of cancer metabolism.

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Growth-Factors for Protozoa

By RICHARD P. HALL

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I. INTRODUCTION

Specific investigations in this field have covered a period of approximately ten years. So far, it has been demonstrated that at least five growth-factors are essential for one protozoon or another, although no single species has yet been shown to require all of these substances. Growth of various species is stimulated by several vitamins not yet known to be essential for these organisms, and there is also evidence that particular vitamins may influence the characteristics of the growth curve. It has been possible to relate one growth-factor to certain phases of metabolism in a few species. While it may be assumed that the activities of several additional vitamins are more or less comparable to those reported in other microörganisms, there is no definite information concerning their rôles in protozoan metabolism. Although a number of Protozoa are now known to require vitamins for growth, it has been found that some of the flagellates may be grown in culture media originally containing no growth-factors.

On the basis of their apparent requirements, Protozoa fall into two groups: those capable of growing in simple media free from growth-factors, and those which can be grown only in media known to contain various growth-factors. Members of the first group are restricted to the plant-like flagellates (*Phytomastigophora*).

It is now established that such chlorophyll-bearing flagellates as *Chlamydo-*

monas agloëformis (1), *Chlorogonium euchlorum* (2, 3, 4), *Lobomonas piriformis* (5, 6), *Euglena gracilis* (7, 8), *Euglena stellata* (9) and *Euglena viridis* (10) can be grown as photoautotrophs.[1] Similarly, certain colorless species —e.g., *Polytoma obtusum* (1, 11) and *Astasia sp.* (12)—may carry on heteroautotrophic nutrition under suitable conditions. Although it is clear that these flagellates are not dependent upon external sources of growth-factors, synthesis of the necessary substances obviously is possible. Consequently, none of the evidence eliminates the possibility that various vitamins may be involved in the metabolism of these flagellates, although assumptions to this effect must remain subject to experimental confirmation.

Since it is not yet possible, on the basis of direct evidence, to reach definite conclusions concerning the synthesis of vitamins by Protozoa, a growth-factor will be termed essential in the following discussion only when the substance must be present in a medium to insure growth of a given species. A non-essential factor, accordingly, will be considered one which need not be present in a suitable medium, even though the substance may stimulate growth when added to the medium. A growth-factor non-essential in this sense either is not required in metabolism, or else is synthesized rapidly enough to meet basic requirements.

II. THE BASAL MEDIUM

Evaluation of the results obtained with Protozoa necessarily involves a consideration of experimental methods. Two general procedures have been followed in the investigation of vitamin requirements. In one method, a strain of Protozoa is carried for a number of transfers in a basal medium deficient in a particular growth-factor. If the missing substance is actually essential the control strain in the basal medium should be lost after several transfers, whereas the basal medium with added growth-factor should support growth indefinitely. The number of serial transfers obviously must be great enough to eliminate any growth-factors carried over with the inoculum from the original stock. On the other hand, the rate of dilution —which is determined by the size of inocula and the frequency of transfer —must not be too rapid, or else multiplication of the Protozoa may fail to keep pace with the dilution and the strain will be lost as a direct result.

A second procedure involves tracing, for only a single transfer, the effects of a vitamin added to a suitable basal medium. Results so obtained will indicate effects upon maximal density of population and rate of growth, but such data cannot be expected to prove that a particular substance is or is not essential to growth. However, with a basal medium so deficient in vitamins that it supports little or no growth, positive effects of added vitamins may furnish significant clues to actual requirements.

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[1] In photoautotrophic nutrition, chlorophyll-bearing organisms carrying on photosynthesis utilize inorganic nitrogen sources and are independent of organic foods. Heteroautotrophic nutrition likewise involves utilization of inorganic nitrogen as the sole nitrogen source, but an organic carbon source is required in the absence of photosynthesis. Under the appropriate conditions, microörganisms may be termed photoautotrophs and heteroautotrophs, respectively.

In all such investigations, the validity of any conclusion rests ultimately upon the nature of the basal medium. Obviously, the ideal medium is a synthetic one containing known nutritive substances but no growth-factors. This ideal has been attained in some of the work on plant-like flagellates (13), but no satisfactory synthetic medium is yet available for the higher Protozoa.

In any case, a suitable basal medium must meet certain requirements, two of the most obvious being that the medium should be harmless to the test organism and must supply essential foods in adequate amounts. Furthermore, the control medium, in actual use, must supply any essential inorganic substances and should contain all essential growth-factors except the ones being tested. Otherwise, the potential effect of a growth-factor might be masked by a deficiency in some other direction. The importance of the mineral requirements must not be underestimated. To mention one example, phosphate is known to be essential both in respiration and in assimilation, and is obviously involved in the activity of thiamin and riboflavin. In addition to possible relationships between particular growth-factors and specific inorganic substances, a given mineral deficiency may, through some other metabolic relationship, limit the size of the population and thus minimize apparent effects of an added vitamin. For instance, Hutchens (14) has shown that the potential effect of thiamin on growth of *Chilomonas paramecium* is limited by a culture medium deficient in iron. It is even conceivable that a failure to supply essential minerals in a basal medium might serve as the stimulus to a search for some unknown new vitamin.

Various types of basal media have been used for the higher Protozoa. In some cases, the growth-factor content of a previously satisfactory medium has been reduced artificially. Such media have been used particularly for investigations on thiamin and cholesterol. For example, dethiaminized media have been produced by heating peptone solutions in the autoclave after adjusting the pH to 9.6 or higher. The efficiency of this procedure depends upon the temperature and the time of treatment. Heating for relatively short periods—e.g., 20–30 minutes at 120–122°C.—does not dethiaminize a peptone solution (15, 16, 17), although the thiamin content may be detectably reduced. Hence, the details of treatment must be considered carefully in basing interpretations upon such a basal medium. A possible complication is also that various constituents of the medium, in addition to the growth-factor under investigation, may be modified by rigorous treatment.

In some instances, the choice has been an untreated medium already inadequate for growth. Although media of this type have obvious advantages, it is difficult to devise untreated basal media which are deficient in particular vitamins and yet adequate in other respects.

As a third choice, a naturally depleted basal medium has been prepared as a filtrate from old cultures of the test organism (18, 19, 20). It might be expected that such media would be specifically deficient in vitamins essential to growth of the organisms concerned, while the available data (19) suggest that the basic food value of the medium is not reduced appreciably.

III. Thiamin

An apparent need for thiamin has been reported in several ciliates and certain parasitic flagellates (Table II). Whether this vitamin plays an essential rôle in the metabolism of all types of Protozoa is yet to be determined, and any attempt at generalization encounters the fact that no plant-like flagellate has yet been shown to require an exogenous source of thiamin (Table I). If it is to be assumed that Protozoa in general do make use of thiamin in metabolism, such a thesis must rest for the present upon the unproven premise that heteroautotrophic and photoautotrophic flagellates synthesize thiamin. Although it is reasonable enough to suspect that these flagellates may synthesize thiamin from simple materials, there are some indications that this synthetic ability, if it actually exists, is not a universal attribute of the colorless or even the chlorophyll-bearing *Phytomastigophora.*

The observations of A. Lwoff and Dusi suggest that the synthetic ability of *Phytomastigophora* may vary even within the limits of a genus. Thus, *Polytoma obtusum* (1, 11) and *P. uvella* (11, 21) appear to be heteroautotrophs, although apparently contradictory findings have been reported for the latter (22). On the other hand, *P. caudatum* (11, 23) and *P. ocellatum* (11, 24) are said to require the thiazole component of thiamin. The related phytomonad, *Polytomella caeca* (11, 25, 26, 27) seems to need both thiazole and pyrimidine. These two substances are not required specifically (25), since thiochrome, 2-methyl-4-amino-5-hydroxymethyl-pyrimidine, or 2-methyl-4-amino-5-thioformylaminomethyl-pyrimidine may be substituted for the pyrimidine constituent of thiamin, while "iso-vitamin B_1" will replace thiazole but not pyrimidine.

Lwoff and Dusi have inferred that thiamin is active in the metabolism of all these flagellates, and that the more fastidious species are unable to synthesize the vitamin except from thiazole or from both components. These conclusions are particularly interesting in that they suggest a progressive decrease in the power of synthesis, leading toward the condition found in higher Protozoa which seem to be completely dependent upon external sources of thiamin.

TABLE I

Requirements of the Plant-like Flagellates (*Phytomastigophora*)

Species; Basal Media	Thiazole	Pyrimidine	Thiamin
Inorganic medium			
Euglenida:			
Euglena anabaena (9, 33)	—	—	—
Euglena gracilis (7, 8)	—	—	—
Euglena klebsii (9)	—	—	—
Euglena stellata (9)	—	—	—
Euglena viridis (10)	—	—	—
Phytomonadida:			
Chlamydomonas agloëformis (1)	—	—	—
Chlorogonium elongatum (2)	—	—	—
Chlorogonium euchlorum (2, 3, 4)	—	—	—
Haematococcus pluvialis (1)	—	—	—
Lobomonas piriformis (5, 6)	—	—	—
Acetate + inorganic medium			
Cryptomonadida:			
Chilomonas paramecium (14, 29, 30)	—	—	—
Chilomonas paramecium (11, 25)	+	+	—
Euglenida:			
Astasia sp. (12)	—	—	—
Euglena gracilis, darkness (34)	—	—	—
Phytomonadida:			
Polytoma caudatum (11, 23)	+	—	—
Polytoma obtusum (1, 11)	—	—	—
Polytoma ocellatum (11, 24)	+	—	—
Polytoma uvella (11, 21)	—	—	—
Polytomella caeca (11, 25, 26, 27)	+	+	—
Asparagine or amino acid + inorganic salts			
Cryptomonadida:			
Chilomonas paramecium (31)	—	—	—
Euglenida:			
Euglena anabaena, light (33)	—	—	—
Euglena gracilis, darkness (11)	—	+	—
Euglena pisciformis, light (32)	+	+	—

+, said to be essential for growth.
—, not essential for growth.

Unfortunately, there is no conclusive evidence that either thiazole or the combination of thiazole and pyrimidine is really essential to growth of these flagellates. Populations of 10,000 to 15,000 flagellates per cc. have been reported in the basal medium alone (11), and serial transfers were possible. Lwoff and Dusi attributed these results to growth-factors supposedly contributed by cotton fibers dropping from the plugs into the culture tubes. This is not necessarily the correct interpretation. Schoenborn (28) eliminated contamination with cotton by using side-arm flasks, the mouths of which were closed with inverted vials, and yet *Astasia* grew just as well as in ordinary flasks plugged with cotton. Furthermore, the attempt of Lwoff and Dusi (11) to eliminate cotton plugs by covering tubes with inverted tubes is open to the criticism that a seal might be formed, with consequent modification of the oxygen tension or other conditions and eventual loss of the cultures. Such a change in technique might be important, since Schoenborn (12) found that growth of *Astasia* was less abundant in tubes than in flasks. In fact, growth sometimes failed in tubes when flasks of the same medium supported growth. In other words, Lwoff and Dusi may have demonstrated merely that growth of their flagellates in the basal medium was retarded by unfavorable conditions, instead of proving that cotton fibers contributed substances essential to growth. Until further evidence is available, the status of thiazole and pyrimidine as essential growth-factors for *Polytoma* and *Polytomella* must remain uncertain.

The case of *Chilomonas paramecium* is similar. A. Lwoff and Dusi (23) first reported that thiazole is required by this flagellate, but concluded later (11, 25) that both thiazole and pyrimidine are needed. These components of thiamin could be replaced by related substances, as in the case of *Polytomella caeca.* In spite of these supposed requirements, the basal medium of Lwoff and Dusi supported growth without added growth-factors. In addition, growth of this flagellate as a heteroautotroph, both in depression-slides (29) and in flasks (14, 30), has been reported. Although Hall and Loefer (31) failed to grow *C. paramecium* as a heteroautotroph in culture tubes, their medium is known to have been deficient in iron (12, 14) which may have been the limiting factor. The use of depression-slides in petri dishes automatically eliminates contamination with cotton fibers. Hence, the evidence fails to show that thiazole and pyrimidine are essential to growth of *C. paramecium.*

Euglena pisciformis is said to require both thiazole and pyrimidine for growth in light (32). Dusi's basal medium, containing asparagine and inorganic salts, failed to support growth beyond the second transfer, but with added thiazole and pyrimidine the medium was satisfactory. Obviously, these substances stimulate growth of *E. pisciformis.* However, the use of small inocula (2 drops) suggests that the high rate of dilution in serial transfers may have precluded positive results with the basal medium (see discussion in section II, p. 250). It may be pointed out that *E. anabaena,* a facultative photoautotroph (9, 33), grows quite slowly in glycine and asparagine media (33), although serial transfers are possible with relatively large inocula and incubation periods of several weeks. Accordingly, the repetition of Dusi's investigations, using larger inocula and less frequent transfers, seems desirable.

Another chlorophyll-bearing flagellate, *Euglena gracilis*, is said to require pyrimidine for growth in darkness (11), although this species grows as a photoautotroph in light (7, 8). This interpretation implies some relation between a need for pyrimidine and the suppression of photosynthesis, although such a relationship does not seem logical in view of the varied requirements of colorless species (Table I). Furthermore, an autotrophic stock, derived from this same strain of *E. gracilis*, has been grown in darkness for 18 weeks (4 transfers) in an acetate and inorganic salt medium (34). Hence, this supposed need for pyrimidine may represent another case in which small inocula and frequent transfers have led to apparent failure with a simple basal medium.

In view of the inconclusive nature of the evidence, it seems premature to conclude that the thiazole and pyrimidine constitutents of thiamin must be supplied for growth of any plant-like flagellate, although these substances undoubtedly stimulate growth of certain species. The entire question requires further investigation, with particular reference to the rate of dilution in the serial-transfer technique.

So far as the higher Protozoa are concerned, it is possible that a need for exogenous sources of thiamin will prove to be a fundamental distinction between these organisms and the plant-like flagellates. Definite evidence that thiamin is essential for growth has been obtained with several species, and *none* of the other higher Protozoa has yet been grown in a medium known to contain no thiamin.

The first conclusive evidence for thiamin requirements among the Protozoa was obtained with certain ciliates. An apparent requirement of the vitamin B complex for growth of *Colpidium campylum* and *C. striatum* was noted some years ago (35). Subsequently, thiamin was found to be essential for both *C. striatum* (15, 36) and *C. campylum* (16, 17, 37). In both cases, the evidence is based upon the serial-transfer method with basal media deficient in thiamin. The conclusion of A. and M. Lwoff (38), that *Glaucoma piriformis* requires thiamin, was not supported by adequate evidence, since their basal medium apparently contained thiamin (16, 17). In a later investigation, however, dethiaminized basal media were used to produce conclusive evidence (39). Dewey (40), in a preliminary report, has concluded that *Tetrahymena geleii* requires thiamin, but the nature of her evidence was not indicated.

In addition to the ciliates, several parasitic flagellates (Table II) need thiamin for growth. M. Lwoff's (41) report, that thiamin is essential for *Leptomonas oncopelti*, rests upon the apparently incorrect assumption that silk-peptone contains no thiamin. However, confirmatory evidence has since been obtained with dethiaminized media (42), and it now appears that *L. oncopelti*, *L. culicidarum* and *L. fasciculata* all require culture media containing thiamin. For these flagellates, thiamin cannot be replaced by several derivatives or by its thiazole and pyrimidine components (42).

TABLE II

Requirements of the Higher Protozoa: Vitamins of the B-complex for Which Data Are Available

Species	Thiamin	Riboflavin	Pyridoxin	Pantothenic Acid
Protomastigida:				
Leishmania agamae (48)	?*	?	?	?
Leishmania ceramodactyli (48)	?*	?	?	?
Leishmania donovani (48)	?*	?	?	?
Leishmania tropica (48)	?*	?	?	?
Leptomonas ctenocephali (60)	?*	?	?	?
Leptomonas culicidarum (42)	+	?	?	?
Leptomonas fasciculata (42)	+	?	?	?
Leptomonas oncopelti (41)	+	?	?	?
Trypanosoma cruzi (47)	?*	?	?	?
Polymastigida:				
Eutrichomastix colubrorum (49, 50, 51)	?*	?	?	?
Trichomonas columbae (51, 54, 56, 57, 58)	?*	?	?	?
Trichomonas foetus (51, 52)	?*	?	?	?
Trichomonas gallinarum (55)	?*	?	?	?
Amoebida:				
Acanthamoeba castellanii (43)	C	?	?	?
Ciliata:				
Colpidium campylum (17, 37)	+	+	?	?
Colpidium striatum	+ (15, 36)	S (15)	S (15)	S (46)
Glaucoma piriformis	+ (39)	S (18)	?	?
Pleurotricha lanceolata (45)	?*	?	?	?
Stylonychia pustulata (45)	?*	?	?	?
Tetrahymena geleii (40)	+	S	S	?

+, said to be required for growth.

?, requirement not established.

*, growth reported only in media which presumably contain thiamin and other vitamins of the B-complex.

C, thiazole and pyrimidine said to be used in synthesis of thiamin, which is not required from exogenous sources.

S, apparently increases density of population.

Acanthamoeba castellanii, presumably an exception among the higher

Protozoa, is said to synthesize thiamin from thiazole and pyrimidine instead of being dependent upon external sources of thiamin as such. A. Lwoff (43) failed to grow this species in a solution of silk-peptone, whereas growth was possible when pyrimidine was added to the medium. Contrary to Lwoff's assumption, his silk-peptone basal medium probably contained thiamin (17). Furthermore, his difficulties with this medium may possibly be traced to rapid dilution in serial transfers, since growth of *A. castellanii* was said to be very slow even with added thiamin, inocula of at least 6 drops being necessary to insure rich cultures. Consequently, Lwoff's results with silk-peptone may indicate merely that pyrimidine accelerates growth of *A. castellanii*. Additional experiments with beef-peptone, heated in alkaline solution, were not described in sufficient detail for evaluation of the evidence from this source.

In a few instances, the relations of thiamin to growth and survival of ciliate populations have been traced in various types of media (18, 19, 20, 44). The available evidence indicates that, when thiamin is added to a basal medium containing a moderate amount of this vitamin but satisfactory otherwise, there is no increase in rate of growth. However, growth of the population is continued beyond the level attained in the basal medium, and maximal density is maintained for a somewhat longer period than in the controls. Upon addition of thiamin to a low-thiamin medium, both density of population and rate of growth are increased. On the other hand, if lack of thiamin is not a factor directly limiting maximal density, the addition of thiamin may produce no increase in population, although the maximal stationary phase may be prolonged to some extent. It remains to be determined just how the population is maintained at a high density for longer periods than in the controls—whether fission is continued throughout the extended stationary phase to replace dying ciliates, or the life of individual ciliates is prolonged. At present, it appears that the available concentration of thiamin is related to the potential density of population and to duration of the maximal stationary phase in cultures of ciliates.

IV. Riboflavin

Very little work has been done on riboflavin requirements of Protozoa. Elliott (15) found that this growth-factor, added to a dethiaminized casein-peptone medium, accelerated growth and increased the population yield of *Colpidium striatum* in the first transfer. The effects were much less striking than those produced by the addition of thiamin, and serial transfers were impossible with riboflavin alone. A comparable effect of riboflavin in combination with thiamin has been observed in *Glaucoma piriformis* (18), and stimulation of growth may also have been observed by Lilly

(45) in *Pleurotricha lanceolata.* In the latter case, however, it is difficult to interpret the results because data for controls in the basal medium were not published. Combinations of riboflavin with thiamin and pyridoxin have been more effective than thiamin alone in increasing growth of *Tetrahymena geleii*, although specific effects of riboflavin were not indicated in the preliminary reports (20, 40).

Serial-transfer tests have indicated that riboflavin is essential for growth of *Colpidium campylum* in a de-ashed gelatin medium (17, 37). As yet, the status of riboflavin as a growth-factor for other Protozoa is uncertain.

V. PYRIDOXIN

Effects of pyridoxin on growth of Protozoa were first investigated by Elliott (15), who obtained a two- to three-fold increase of *C. striatum* in the first transfer by adding a pyridoxin concentrate to a dethiaminized basal medium. Lilly (45) may have observed a similar effect on *Pleurotricha lanceolata*, although the omission of data for controls leaves the reader with no standard of comparison. Combinations of pyridoxin with thiamin and riboflavin also have been tested with *Tetrahymena geleii* (20, 40), but the specific effects of pyridoxin are yet to be described. At present, there seems to be no evidence that pyridoxin is essential to growth of Protozoa.

VI. PANTOTHENIC ACID

Detailed experiments with synthetic pantothenic acid have not yet been reported for Protozoa. However, Elliott (46) found growth of *C. striatum* to be accelerated by a concentrate of this growth-factor. The effect, noted in short-run experiments with casein-peptone medium, was limited to the pH range 5.5–6.5. Pantothenic acid produced no significant effect when added to a gelatin medium. Dewey (40) combined pantothenic acid with other vitamins in her work with *T. geleii*, but did not present detailed data. At present, there is no conclusive evidence that pantothenic acid is required for growth of Protozoa.

VII. OTHER VITAMINS OF THE B-COMPLEX

Nicotinic acid (45), its amide (18, 19) and a biotin concentrate (40) have been tested with different ciliates, but the relations of these substances to growth are not clear. Nicotinic acid, as used by Lilly (45), seems to have been toxic for *Stylonychia pustulata*, while the data for *Pleurotricha lanceolata* are inconclusive.

VIII. ASCORBIC ACID

Several parasitic flagellates, representing the orders Protomastigida and Polymastigida, are said to require ascorbic acid for growth (Table III).

Trypanosoma cruzi, for example, has been grown by M. Lwoff (47) in a peptone and agar medium enriched with serum, hemin and ascorbic acid, but not without the ascorbic acid. On the basis of similar evidence, *Leishmania tropica* also seems to need ascorbic acid. M. Lwoff (48) has noted that species of *Leishmania* differ with respect to this requirement—*L. agamae* and *L. ceramodactyli* were grown in a medium containing peptone,

TABLE III

Requirements of the Higher Protozoa: Growth-factors Other than the Vitamin B-complex

Species	Ascorbic Acid	Hematin	Cholesterol	Unidentified Factors
Protomastigida:				
Leishmania agamae (48)	−	+	?	+
Leishmania ceramodactyli (48)	−	+	?	+
Leishmania donovani (48)	+	+	?	+
Leishmania tropica (47, 48)	+	+	?	+
Leptomonas ctenocephali (60)	?	+	?	?
Leptomonas culicidarum culicis (62)	?	−	?	?
Leptomonas culicidarum anophelis (62)	?	+	?	?
Leptomonas fasciculata (60, 62)	?	+	?	?
Leptomonas media (62)	?	−	?	?
Leptomonas muscidarum (62)	?	+	?	?
Leptomonas oncopelti (41, 42, 62, 63)	?	−	?	?
Leptomonas parva (62)	?	−	?	?
Leptomonas pyrrhocoris (62)	?	+	?	?
Trypanosoma cruzi (47)	+	+	?	+
Polymastigida:				
Eutrichomastix colubrorum	+ (50)	?	+ (50, 51)	+ (50)
Trichomonas columbae	+ (54)	?	+ (51, 56, 57)	?
Trichomonas foetus	+ (51, 52)	?	+ (52)	+ (52)
Trichomonas gallinarum (55)	+	?	?	+
Amoebida:				
Acanthamoeba castellanii (43)	?	?	?	?
Ciliata:				
Colpidium campylum	?	?	?	?
Colpidium striatum	?	?	?	?
Glaucoma piriformis	?	?	?	?
Pleurotricha lanceolata (45)	?	?	?	+
Stylonychia pustulata (45)	?	?	?	+
Tetrahymena geleii (40)	?	?	?	+

+, reported to be essential for growth.
−, exogenous source not required.
?, requirement not established.

serum and hematin, whereas both *L. donovani* and *L. tropica* required ascorbic acid in addition. In view of the complex basal media used by M. Lwoff, such differences may be only quantitative, but the question cannot be settled without further information.

The observations of Cailleau (49, 50) on *Eutrichomastix colubrorum* are interesting. This flagellate was grown in an enriched peptone medium to which ascorbic acid was added in a concentration of "1×10^{-4}" (presumably grams per cubic centimeter), whereas serial transfers were impossible with concentrations of 1×10^{-5} or less. Such results are unusual in that other vitamins are effective for Protozoa in much lower concentrations. Ascorbic acid could be replaced (49) by fragments of liver, by serum (1:20), by *d-iso*-ascorbic acid, *d*-gluco-ascorbic acid, reductone (glucic acid), reductinic acid, or *d*-gluco-hepto-ascorbic acid. Serum lost its activity after storage for five months at room temperature; liver, after storage for 14 months.

In the case of *Trichomonas foetus* (52), the evidence that ascorbic acid is essential rests upon failure to grow the flagellate in broth enriched with beef serum, unless ascorbic acid was added. However, it should be noted that Lyford (53) obtained good growth of *T. foetus* in dextrose-broth and rabbit serum.

For *Trichomonas columbae* (54), peptone with added serum (1:20) formed an adequate medium. Serum aged for five months at room temperature was ineffective, but the combination of aged serum and ascorbic acid insured growth. Comparable results were obtained with a peptone and liver medium. *T. gallinarum* (55) failed to grow beyond the third transfer in Locke-egg-serum medium unless ascorbic acid was supplied.

The data of Cailleau may indicate that *Eutrichomastix colubrorum* and several species of *Trichomonas* all require ascorbic acid as a growth-factor. On the other hand, the apparent need for such high concentrations of this vitamin suggests another possibility. *E. colubrorum*, for example, develops best under conditions of "semi-anaerobiosis" (51). Since ascorbic acid is known to exert reducing action *in vitro*, it is possible that a non-specific effect on the redox potential of the medium may explain the apparent necessity for such high concentrations of this vitamin. An effect of this kind would be particularly important with small inocula, and Cailleau (51) seems to have inoculated cultures with only a few drops of material. In this connection, it may be significant that Lyford (53) found growth of *T. foetus* to be dependent upon size of the inoculum—with small inocula, the maximal density of population was much lower than that reached in cultures receiving large inocula. Favorable effects on growth of *Eutrichomastix* and *Trichomonas*, comparable to the action of ascorbic acid in relatively high concentration, might be produced by thioglycolic acid or other substances not classified as vitamins. Consequently, possible nonspecific relations between ascorbic acid and a favorable redox potential of culture media should be considered before this vitamin is recognized as an essential growth-factor for *E. colubrorum* or other Protozoa.

IX. STEROLS

Several parasitic flagellates seem to require cholesterol or other sterols as growth-factors (Table III). One of these organisms is *Trichomonas columbae* (51, 56, 57, 58), which has been grown in peptone broth containing fragments of liver. The activity of liver was lost after extraction with acetone, but was restored by the addition of cholesterol. Highly purified samples of cholesterol were effective, so that impurities obviously were not involved. Cailleau (51) has attempted to correlate chemical structure of 66 different sterols with activity as growth-factors for *T. columbae.* Activity comparable to that of cholesterol was exhibited by cholestanol, allocholesterol, 22-dihydroergosterol, sitosterol, cinchol, ergostanol and several other sterols. Ergosterol was moderately active, provided the substance was not sterilized by heat; however, irradiated ergosterol was inactive. The effect, therefore, has presumably nothing to do with vitamin D. Later observations have indicated that cholesterol also serves as a growth-factor for *T. foetus* (52) and *Eutrichomastix colubrorum* (50).

X. HEMATIN AND RELATED SUBSTANCES

That species of *Trypanosoma* and various related flagellates apparently require culture media containing blood was first noted many years ago. Salle and Schmidt (59) found that, for *Leishmania tropica,* blood could be replaced by a solution of hemoglobin, and they suggested that hemoglobin might serve as an accessory growth-factor. This possibility has since been considered by M. Lwoff (48, 60, 61, 62). Several species of *Leptomonas*—e.g., *L. oncopelti* (63)—have been grown in peptone media without added blood; certain other species of *Leptomonas,* as well as species of *Leishmania* and *Trypanosoma,* are more exacting in their requirements (Table III).

Both *Leptomonas ctenocephali* and *L. fasciculata,* for example, were found to require blood, the requirements of the former being much higher than those of the latter (60). Hematin, which showed much greater activity, proved to be an excellent substitute for blood. Since it was possible to replace hematin by Wolff's artificial peroxidase (colloidal Prussian blue) stabilized with gum arabic and to maintain *L. fasciculata* for eight transfers, M. Lwoff concluded: "Le fer actif est l'élément actif de l'hématin et peut, à condition d'être stabilisé par un colloïde protecteur, remplacer le sang pour la culture des Trypanosomides" (60). This conclusion might imply that hematin is not a true growth-factor, since "active iron" in inorganic form apparently served the same purpose.

A few months later, however, it was found that Wolff's "peroxidase" had no effect upon respiration of *L. fasciculata* (61). In the light of this discovery, new attempts were made to grow *L. fasciculata* with Wolff's artificial peroxidase as a substitute for hematin. The results were summarized as follows: "Il m'a été absolument impossible, quelle que soit la quantité de peroxydase ajoutée, d'obtenir le développement d'une culture" (61). These later findings led to the conclusion that protohemin, which may be replaced by protoporphyrin or hematin, is essential for growth of *L. fasciculata*. More recently, it has been reported that hemin is essential for certain other species of *Leptomonas* (62), for *Trypanosoma cruzi* (47) and for four species of *Leishmania* (48).

The relations of protohemin and protoporphyrin to protozoan metabolism have been considered (64, 65, 66). The first step was the demonstration (64) that the rate of respiration in *L. fasciculata* is increased by the addition of whole blood, hematin, protohemin or protoporphyrin to a basal medium, and that the relationships are quantititive. These and other findings led to the conclusion (65, 66) that protohemin is utilized in the synthesis of cytochrome, cytochrome oxidase, catalase and peroxidase. Although cytochrome has been demonstrated in several *free-living* Protozoa, a need for hemin has not been demonstrated in such species.

XI. UNIDENTIFIED GROWTH-FACTORS

It is obviously possible that there may be additional growth-factors, as yet unknown, which are essential in protozoan metabolism. Attempts to demonstrate such new growth-factors have already been reported. Dewey (40), for instance, has concluded that *Tetrahymena geleii* requires perhaps two new factors "in addition to thiamin and possibly riboflavin or other known vitamins." Dewey's non-toxic basal medium—"vitamin-free" casein in 1 per cent solution—failed to support growth of the ciliates beyond the first or second transfer, although a solution of crude casein was quite satisfactory. Combinations of thiamin and other vitamins, added to the basal medium, stimulated growth to some extent, but Dewey did not state that serial transfers were possible. On theoretical grounds, it seems likely that, even after the addition of all the known vitamins which might be essential, Dewey's purified casein medium would not support maximal growth of *T. geleii*, since there was no attempt to supply inorganic substances necessary for growth or for the activity of vitamins. In short, there is no indication that Dewey's medium meets the requirements for a satisfactory basal medium, and none of her preliminary evidence eliminates the possibility that her new "growth-factors" may be nothing more than inorganic substances important in protozoan metabolism.

Another case of supposedly new growth-factors, required by *Pleurotricha lanceolata* and *Stylonychia pustulata*, has been reported by Lilly (45). In his critical experiments, Lilly used as a basal medium a suspension of *Tetrahymena geleii* (as the food supply) in distilled water. To this medium were added thiamin and other vitamins. Although Lilly stated that "media containing physiologically important salts . . . did not support growth," he apparently dismissed the possibility that any of these salts might be essential to the activity of vitamins. At any rate, it was observed that a "standard yeast medium," added to the basal medium, supported better growth of *P. lanceolata* than did the vitamin solutions. The tests apparently were carried through only one transfer. Critical comparison of the vitamin tests with the results for "standard yeast medium" is invalidated by the failure to consider the mineral requirements of *P. lanceolata* in devising a basal medium, by the use of vitamins in concentrations which may have been excessive for the small populations involved, and by the fact that "standard yeast medium" supported growth of *T. geleii* (the basal food supply) whereas the vitamin solutions did not. The tests with *S. pustulata* suggest that the vitamin solutions were mildly toxic as compared with "standard yeast medium" in dilutions which failed to support growth and were "practically equivalent to distilled water as far as their content of the supplementary growth-factor is concerned." These results demonstrate either that the basal medium was unsatisfactory for *S. pustulata* or that the vitamins were used in excessively high concentrations. In either case, the procedure does not furnish an adequate test for the existence of a new growth-factor, and Lilly's investigations should be repeated with a satisfactory basal medium.

An unidentified "factor TS" (47) is said to be essential for growth of *Trypanosoma cruzi*. M. Lwoff has suggested that this substance, present in serum but apparently not in peptone, may be cholesterol; however, identification has not been completed. An unidentified factor, present in serum and possibly this same factor TS, likewise is required by *Leishmania agamae*, *L. ceramodactyli*, *L. donovani* and *L. tropica* (48).

Trichomonas foetus (52) apparently requires a growth-factor which is supplied by living dysentery bacteria (*Shigella dysenteriae*) but not by the bacteria killed at 60°C., and is present in certain samples of serum but not in others. *Eutrichomastix colubrorum* (50) seems to need two unidentified factors in addition to cholesterol and ascorbic acid. One has been supplied in fresh egg-albumin; the other, in a certain peptone (5C of Vaillant) and in guinea-pig liver. *T. gallinarum* (55) also requires a factor present in fresh egg-albumin. The investigations of Cailleau have not yet furnished clues to the identity of any of these factors.

XII. FACTORS INDUCING EXCYSTMENT

In addition to the known or suspected relations of growth-factors to growth and respiration of Protozoa, it is now established that such processes as excystment may be induced by analogous substances active in very low concentrations. Barker and Taylor (67) were the first to demonstrate that excystment of *Colpoda duodenaria* ("*C. cucullus*") may be induced specifically by certain animal or plant extracts, added to basal media in small amounts. These workers suggested that excystment is induced by "some specific substance or a very restricted group of substances possessing specific properties" and that such factors are active even in dilutions of 1:100,000,000. Subsequently, several investigators have attempted to isolate and identify these factors.

Thimann and Barker (68) obtained two active concentrates from hay extract—a weak acid, soluble in chloroform and ether, and a second substance insoluble in organic solvents. Either concentrate induced excystment of *Colpoda cucullus*, but complementary effects were observed with combinations of the two. Haagen-Smit and Thimann (69) later traced the activity of hay infusion to salts of simple organic acids, such as malic, citric, acetic, fumaric and tartaric. The activity of single salts and of mixtures was increased about 400 per cent by an ether-insoluble fraction of hay, which in itself was almost inactive. A comparable co-factor action was obtained with a mixture of pure sugars in dilute solution.

More recently, Prater and Haagen-Smit (70) have isolated two crystalline, non-acidic, non-carbohydrate substances from corn leaves and tops. These substances, in the presence of suitable co-factors, are active in concentrations of $2.0–4.0 \times 10^{-8}$ g. per cc. The co-factors were supplied in certain fractions (III and X) of the corn extract, which could be replaced "at least in part by a carbohydrate solution and suitable combinations of adenylic acid, nicotinic acid, nicotinamide, potassium citrate and malate, and vitamin B_1." A combination of thiamin, potassium citrate and potassium malate apparently was more active than a mixture of fractions III and X. These co-factors, tested alone, were without appreciable effect on excystment.

In view of these interesting observations on excystment, Taylor and Strickland (71) have suggested that the formation of resting cysts may involve the inactivation of certain enzyme systems through loss of co-enzymes or complementary components, inactivation being induced by some product of the ciliates. It would be interesting to determine whether the formation of resting cysts can be prevented by any of the specific excystment-inducing substances or their co-factors. Future investigations should be facilitated by the recent establishment of *C. duodenaria* in pure culture (72).

XIII. Miscellaneous Growth Stimulants

Plant growth "hormones", or auxins, have been tested on Protozoa in one instance (73); (see also (68) for experiments on excystment). Growth of *Euglena gracilis* was accelerated by these substances, but there was no favorable effect on *Khawkinea halli*, a colorless euglenoid flagellate, or on the ciliate, *Colpidium striatum.*

Pimelic acid, which may serve as a growth-factor for certain bacteria (74), has been found to accelerate growth of *Colpidium campylum* (75). However, there is no evidence that this substance is essential to growth of ciliates (17).

XIV. Discussion

Investigations on the vitamin requirements of Protozoa have been confined to a few laboratories, with the result that progress has been slow and published data are not extensive. In addition to the many problems which remain untouched or unrecognized, some of the questions which have been considered experimentally are still confused by inadequate or contradictory data. For instance, there is as yet no conclusive evidence that any protozoon synthesizes vitamins, although many workers might be inclined to take such a proposition for granted so far as the plant-like flagellates are concerned. Even for some of these flagellates, however, there are unconfirmed indications that at least the thiazole and pyrimidine components of thiamin may be required from exogenous sources. This question should be settled, since it has a bearing on the physiological evolution of Protozoa and upon fundamental distinctions between the *Phytomastigophora* and the higher Protozoa. Among the latter, no single species has been investigated exhaustively, and at most no more than two identified growth-factors have been reported as essential for any one organism. The metabolic relationships of growth-factors in Protozoa have been considered critically only in the case of hematin (section X, p. 261). While observations on other organisms furnish bases for assumptions in protozoan physiology, hypotheses are unsatisfactory substitutes for specific information on the Protozoa.

The accumulation of conclusive data has been retarded to some extent by inadequate technical procedures. Some difficulties are unavoidable with the present lack of synthetic media suitable for the higher Protozoa. On the other hand, the use of basal media containing relatively large amounts of the growth-factor under investigation, the failure to supply essential inorganic salts in basal media, and excessively rapid dilution in the serial-transfer technique have all contributed to occasional confusion in the interpretation of experimental results. Such errors indicate the need for applying certain basic principles to the investigation of protozoan requirements. Although the following generalizations are certainly obvious ones, it may be pointed out that recognition of some such criteria would prevent repetition of certain avoidable mistakes in technique.

(a) The basal medium must be non-toxic, must contain an adequate food supply, must satisfy mineral requirements of the organism, and for the higher Protozoa should contain all essential growth-factors except those being tested. Temperature, pH of the medium and other environmental conditions should be reasonably suitable, although not necessarily optimal for the test organism.

(b) In determining vitamin requirements of Protozoa, reliance must be placed mainly upon the serial-transfer technique and not upon cultures incubated for only one transfer. Furthermore, serial dilution must not be too rapid—in other words, relatively large inocula and long incubation are often preferable to small inocula and short incubation periods.

(c) Mere stimulation of growth, which may not be specific, should not be confused with the requirement of a particular vitamin for growth. Essential growth-factors may and often do stimulate growth, but a growth stimulant is by no means always an essential growth-factor. Confusion may arise, for example, when a basal medium supports rapid growth of a test organism with an added growth-factor but only slow growth without it. Such data would not constitute conclusive evidence that the particular factor is essential to growth.

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Physiology of Anti-Pernicious Anemia Material

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I. CLINICAL ASPECTS OF PERNICIOUS ANEMIA

Pernicious anemia is a disease due to deficiency in the body of material derived from food and manifest as a macrocytic anemia associated with disturbances of the gastro-intestinal tract, and frequently of the neural system. If untreated the disease progresses over a period of usually about three years to a fatal issue. Under adequate treatment with suitable preparations of liver or stomach, the blood remains essentially normal, symptoms but not all signs of disorder of the digestive system vanish, and no progress or development of neural system lesions occurs.

In order to understand the physiology of anti-pernicious anemia material it is necessary to point out briefly some of the clinical aspects of the disease. The condition occurs particularly in the fourth and fifth decades of life and is rare before the age of thirty. It is prone to develop in blue-eyed individuals of the Nordic race, whose hair has turned prematurely gray. The occurrence of the disease in near relatives of the patient is not rare. The onset is usually insidious and the symptoms of anemia *per se* are seldom obvious until the hemoglobin has fallen below 65 per cent. Gastro-intestinal symptoms may be the initial ones. Recurrent burning and soreness of the tongue is frequent. The red, inflamed surface with vesicle formation leads to the smooth, shiny, atrophic tongue characteristic of the disease. A similar process occurs in the esophagus and stomach. Achlorhydria is a feature of the disease. The neural manifestations may be present long before there is clear-cut evidence of macrocytic anemia. They may be limited to paresthesias of the hands and feet or may be those of severe damage to the posterior and lateral columns of the spinal cord. Diminution of vibration sense is one of the earliest and commonest signs of the latter. Peripheral nerve involvement also occurs.

1. Blood Picture

The blood picture in well-developed pernicious anemia is typical of diminished activity of the bone marrow with decreased numbers of white blood cells and blood platelets as well as of red blood cells. The number of red blood cells is usually below 2,500,000 per cu. mm. The mean corpuscular volume of the red blood cells is large (110 to 160 cu. micra). Some increase in size is prone to occur before any outspoken reduction of the red cell count. The mean corpuscular hemoglobin concentration is 32 per cent or more, and the color index almost always is above 1. The plasma pigments are usually increased with a positive indirect van den Bergh reaction.

The red cells in the untreated patient may show practically any of the abnormalities known to occur, except pronounced achromia. A large percentage of deeply stained oval cells, usually macrocytes, is a particular feature. Cells smaller than normal and tiny microcytes are also seen, so that distinctly marked variation in size often occurs. All forms of abnormal shapes may be present, the most bizarre being among the medium-sized and smaller cells. Diffuse and punctate polychromatophilia is common but reticulocytes above 5 per cent are unusual in the untreated patient. A variety of nucleated red blood cells may be seen. With the decreased numbers of white cells the lymphocytes are usually increased. Occasionally the eosinophils are moderately increased. They may reach over 50 per cent after raw liver feeding but not after cooked liver or oral liver extract therapy. The platelets, although generally diminished, very rarely fall below 40,000 per cu. mm.

The typical blood cell picture is easily recognized but when cases are seen early or in remission, changes in the blood may be absent or slight. The finding of a high color index and a distinctly increased mean corpuscular volume, however, does not necessarily indicate pernicious anemia, since other types of macrocytic anemia occur, which are unrelated to pernicious anemia.

2. Bone Marrow

Pepper and Cohnheim (1, 2) recognized the increased cellularity of the bone marrow, but the studies of Zadek (3) and Peabody (4) have suggested a reason for the paradoxically increased cellularity of the bone marrow as consistent with diminished blood production. In the severely anemic patient the active bone marrow is always increased in amount and may extend throughout the long bones. This dark red marrow shows under the microscope great proliferation of primitive red cells, many of which show mitoses. The primitive cells often appear in large sheets obliterating other structural details so that the white cell and platelet forming centers are fewer than normal and the areas occupied by fat cells greatly reduced or absent.

Fundamental to a consideration of the physiology of the anti-pernicious anemia material is a knowledge of the normal process of blood formation. There is much suggestive clinical evidence to indicate that the effective factor controlling the level of blood formation is the oxygen content of the blood. An increased number of circulating red blood cells is to be found in essentially every condition associated with a diminished oxygen content of the blood if there are no factors preventing normal erythropoiesis. It is apparent that in every case of anemia, the stimulus to erythropoiesis in the form of a diminished oxygen content of the blood must exist. The anemia, however, continues not because of a lack of a bone-marrow stimulus but because of some factors preventing the bone marrow from responding normally to this stimulus, or because the extent of blood loss or destruction is such that it is beyond the capacity of the bone marrow to accommodate thereto.

In pernicious and the related macrocytic anemias, study of the bone marrow shows a degree of primitive cell proliferation in direct proportion to the severity of the anemia. It would appear, therefore, that the stimulus of anoxemia is effective in producing the development of the capillary endothelium into primitive blood cells and that these are stimulated to great proliferation. However, in spite of the continued presence of the stimulus, maturation into erythroblasts and adult erythrocytes apparently does not occur until the anti-pernicious anemia principle is supplied. When this is done there is rapid maturation of the primitive cells through the normoblastic stage into reticulocytes and mature erythrocytes. That this is due to the supplying of a specific deficiency and not to a general stimulus to maturation is suggested by the fact that liver extract has been shown not to influence the rate of division of the red blood cells in chick blastoderms (5). Within a matter of weeks or at most two months the bone marrow of an adequately treated patient with pernicious anemia returns to an essentially normal state.

II. THE EFFECT OF ANTI-PERNICIOUS ANEMIA MATERIAL ON THE BLOOD

1. *The Reticulocyte Response*

When the anti-pernicious anemia material is administered to a patient with pernicious anemia in relapse, the first significant change in the blood is an increase in the circulating reticulocytes. It is clear that the reticulocyte is not a degenerating form but is a red cell intermediate in age between the nucleated and the fully adult type, and the changes in the numbers of reticulocytes in the peripheral blood may frequently serve as a measure of bone marrow activity. Reticulocyte responses to the administration of specific substances occur not only in pernicious anemia but in the anemias due to a deficiency of iron and perhaps other substances. Reticulocyte increases may be produced by material that does not regularly cause an increase in the number of red blood cells, as for example, arsenic by mouth in the form of Fowler's solution (potassium arsenite), or sterile milk protein parenterally injected. The character of the reticulocyte response to such materials is generally quite different from that obtained by the administration of specific substances in anemias due to specific deficiencies. The non-specific type of response is generally irregular and much more prolonged than the characteristic specific responses described below.

There are certain general features of the reticulocyte response in pernicious anemia which obtain if the red blood cell count is below 3,000,000 per cu. mm., when active anti-pernicious anemia material is given orally or parenterally, in a single large dose or in small uniform daily dosage, provided the amount of material given is sufficient to produce a significant increase of reticulocytes (Fig. 1). Almost always within a period of from two to seven days the percentage of the reticulocytes in the peripheral blood begins to increase and progresses regularly to a maximum value within a period of from three to ten days thereafter. Then the reticulocyte percentage declines steadily over a period of time (usually somewhat longer than that taken for the increase), until it returns to a level close to its original value. When the reticulocyte counts are averaged from observations on several patients given similar amounts of active material, an approximately triangular course of varying height and width is usually observed. The peak reticulocyte value may be approximately the same when determined by single counts on two successive days. More frequent counts near the maximum of the curve often show slightly higher intermediate values which restore the acute apex of the triangle. During the reticulocyte response the age of the reticulocytes, as judged by the amount and character of their granular filamentous substance, shows a progressive transition identifiable as a metamorphosis from young to older forms. Characteristically the increase in the total red blood cell count may be accounted for up to the peak of the reaction by the increment of reticulocytes. Thereafter, with continued therapy, the red blood cells increase largely as a result of the delivery of mature erythrocytes. With reticulocyte responses due to small amounts of effective material, the total red blood count may be little increased or may even fall slightly, owing perhaps to an increase of blood volume or to blood destruction. Indeed, the amount of active principle necessary to cause a maximal reticulocyte response may be less than that required to produce a maximal rate of rise in the red blood cells. This difference is due to the fact that daily oral doses of material derived from 300 to 400 g. of liver may in individual instances produce apparently maximal reticulocyte responses but a less than maximal rate of rise of the red blood cells. Analysis of the results obtained in many patients, however, demonstrates that, to produce maximal reticulocyte response a dose larger than the amount derived from 300 g. of liver is often necessary. It is possible that certain very large reticulocyte increases resulting from the parenteral injection of certain liver extracts, and not associated with very significant increases in the number of red blood cells, may be in part due to non-specific reticulocytogenic material present in the extract.

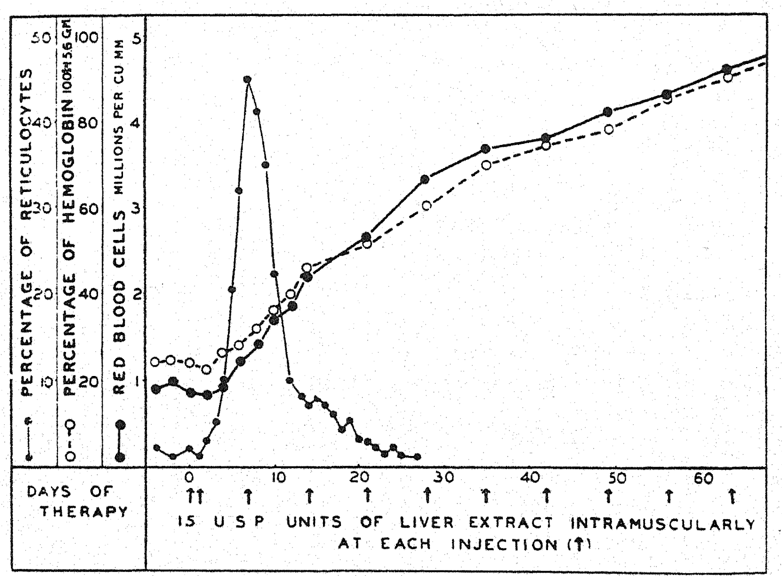

Fig. 1. Characteristic response of the blood to liver extract in a case of pernicious anemia

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In *uncomplicated* cases given what have been considered maximum amounts of potent material, the red cells rise *on the average* at very nearly the same rate, no matter whether the substance is given intravenously, intramuscularly, or by mouth. In four weeks the red blood cells increase, on the average, from the vicinity of 1.4 million/cu. mm., by about 2.5 million or slightly more. To be sure, in a series of cases selected at random the average increase of red cells for those given the material parenterally will be somewhat greater because some of the cases treated orally may have difficulty in absorbing or utilizing the material from the gastro-intestinal tract, and because the parenteral dosage commonly used tends to be relatively greater than amounts given orally.

In pernicious anemia, as the red blood cells rise in response to the administration of active principle, their increase in number frequently outstrips the rate of hemoglobin regeneration and the changes in physical character of the red blood cells. The characteristically increased mean corpuscular volume becomes normal or less, and the mean corpuscular hemoglobin concentration and color-index tend to fall, sometimes below normal values.

The details of the onset, duration, course, and amount of the reticulocyte reaction in pernicious anemia depend particularly upon (1) the initial red blood cell level; (2) the amount of active material given; (3) the portal of entry to the body; (4) the rate at which the material enters the body; and (5) the reactive state of the bone marrow. In the ensuing discussion each factor will be considered in turn, with the assumption that the other four are average and uniform. In the actual interpretation of a given response all the factors must be evaluated.

2. Effect of the Initial Red Blood Cell Level

If a large uniform daily dose of active material is given to each of several patients with pernicious anemia, an inverse relationship between the peak value of the reticulocytes and the initial red blood cell count is observed. This fact is shown by the data plotted in Fig. 2. The relationship between the initial red blood cell level and the maximal percentage value of the reticulocytes during their response holds irrespective of the source of the material or the method of its administration. It is, of course, not proper to compare the absolute values of the reticulocytes at the peak of their rise when the rises are produced by different types of material or different methods of administration. However, the same material given in identical dosage demonstrates the inverse ratio. In patients with over 3 million red blood cells/cu. mm., the reticulocyte responses become variable and may not appear despite a significant effect upon the total red blood cell count. It is probable that under these circumstances the demand of the anemia upon the bone marrow is not sufficient to cause delivery of immature cells into the blood stream, or that the proliferation of the primitive red blood cells in the bone marrow is not sufficient to provide a large amount of tissue for rapid maturation.

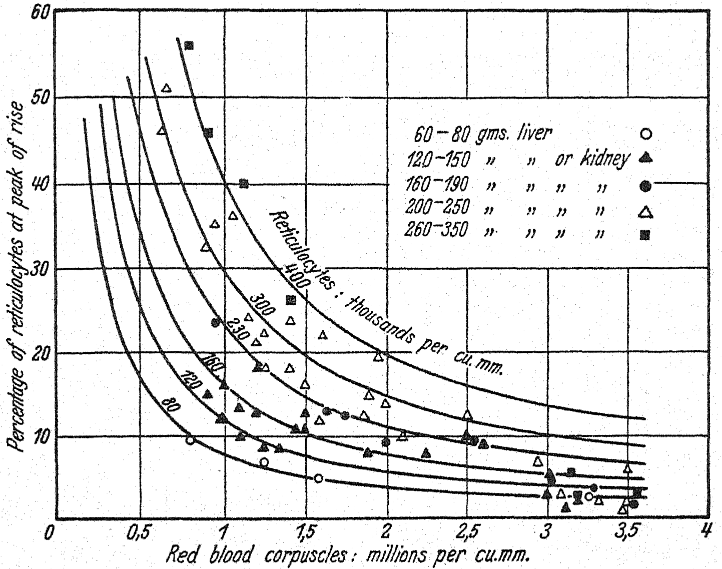

Fig. 2. Height of the reticulocyte peaks in the responses of individual cases of pernicious anemia to the daily administration of the amounts of liver or of kidney indicated, plotted against the red blood cell level existing at the beginning of treatment

Note that there is an inverse relationship between the red blood cell level and the height of the reticulocyte response, and a direct relationship between the latter and the amount of material administered. [Reprinted by permission from *Am. J. Med. Sci.* **175,** 591 (1928).]

3. Effect of the Amount of Potent Material

If to each of several patients with pernicious anemia and with the same initial red blood cell level various amounts of effective material are administered in uniform daily dosage, it appears that there is a direct relationship between the amount of material given and the peak value of the reticulocyte response. This effect, however, approaches a maximum at a decreasing rate as the amount of active material is increased, as shown in Fig. 3. Thereafter an increase of the amount of active material produces no greater increase of the peak value of the reticulocytes but may accelerate the rate of increase of the red blood cell concentration.

4. *Effect of the Route of Administration of Potent Material*

The effectiveness of liver extract when rectally administered is very slight compared with its activity upon oral administration, according to a few observations. If the same amount of a given active material is administered in uniform daily doses by mouth and by intramuscular injection in patients with comparable degrees of anemia, striking differences in the effectiveness of the material are observed. The most accurate comparisons have been made between the effectiveness of the daily oral administration of the liver fraction "G" of Cohn *et al.* (55) and of the daily intramuscular injection of a dilute aqueous solution of the same material. Although the data are scanty, it is evident that the effect of the *daily* oral administration of the extract derived from 600 g. of liver is usually no greater than the effect of the *daily* parenteral administration of the same

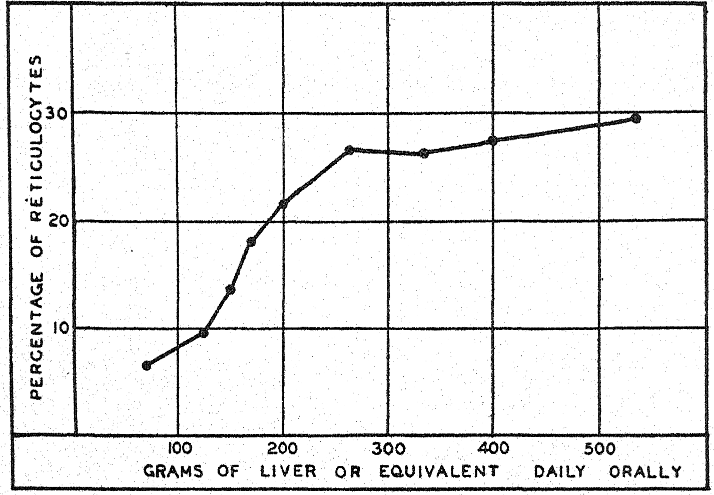

Fig. 3. Relationship between the dosage and the height of the reticulocyte response to the daily oral administration of liver or of liver extract in a hypothetical case of pernicious anemia with an initial red blood cell level of 1.25 million per cu.mm.

The data are derived from observations with liver pulp or with a liver extract conserving about two-thirds of its potency in 99 patients with initial red blood cell levels of between 1 and 2 million per cu.mm. The expected responses in the hypothetical patient with an original red blood cell level of 1.25 million per cu.mm. were calculated when necessary by interpolation, using the slope of the curve shown in Fig. 2 most closely approximating the data of each observation. The averages so obtained from the daily administration of the amounts of liver or equivalent extract indicated are shown by the individual points on the line. [Reprinted by permission from the *Lancet* **1935, 2,** 319 (Aug. 10).]

material derived from 10 g. of liver. Data from certain observations leading to this conclusion are plotted in Fig. 4. It is, therefore, probable that material administered by the parenteral route is at least sixty times as effective as when given by mouth to the average patient. In certain patients, owing possibly to difficulty in the absorption of orally administered material, this ratio is apparently increased. The existence of an intestinal barrier is probably to some extent a physiological phenomenon in regard to the absorption of material in liver extract, iron, and other substances. Whether in certain patients with pernicious anemia increased difficulty in absorption entirely accounts for the relatively slight effects of the usual oral doses of liver extract has not been finally established. There is evidence that the effectiveness of equal amounts of liver extract given intramuscularly in different patients is also variable.

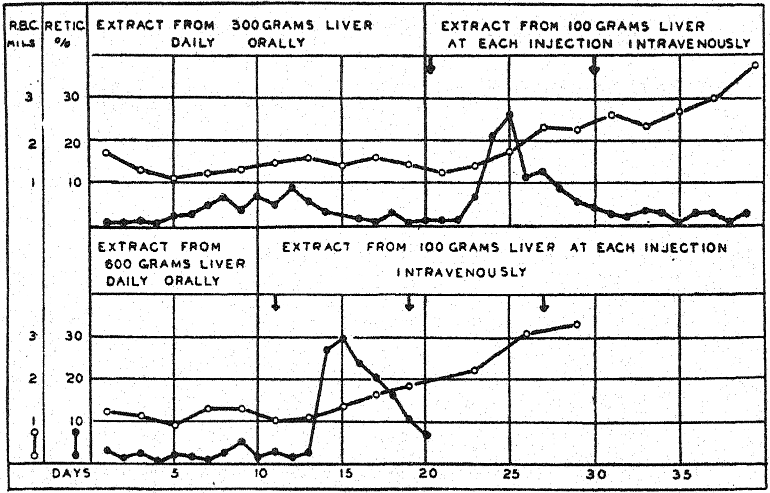

Fig. 4. Comparison of the effectiveness of a liver extract upon oral and parenteral administration in two cases of macrocytic anemia

In the upper part of the figure is shown the relativly slight reticulocyte response and effect upon the red blood cell level produced by the daily oral administration of a liver extract derived from 300 g. of liver, followed by a striking response to a single intravenous injection of the same material derived from 100 g. of liver.

The lower part of the figure shows the negative response in a second patient to the daily oral administration of an extract derived from 600 g. of liver, followed by a striking reticulocyte and red blood cell response after a single intravenous injection of the same material derived from 100 g. of liver. Note, however, that the peak of the reticulocyte response (30 per cent) to the latter is not so great as would be expected (47 per cent). This fact suggests that the failure of the patient to react to the orally administered material is not due entirely to difficulty with absorption, but depends to some extent upon ineffectual internal utilization. [Reprinted by permission from *J. Am. Med. Assoc.* **96**, 1198 (1931), and *Am. J. Med. Sci.* **182**, 741 (1931).]

5. Effect of the Rate of Administration of Potent Material

The rate at which the active principle reaches the bone marrow evidently produces certain modifications in the reticulocyte response. In general the effect of a single massive dose of orally or parenterally administered material is to accelerate the appearance of increased numbers of reticulocytes in the blood stream and to increase the peak value. The tendency for large uniform daily doses of material to cause earlier reticulocyte responses than smaller doses also acts in a similar way. On the contrary, the daily administration of small doses of active principle tends to produce a prolonged low curve of the reticulocyte increase with a delayed and not very acute apex. In the case of parenterally administered liver extract, perhaps especially when given intravenously in a single large dose, there may be an additional non-specific or irritant effect possibly augmenting the production of reticulocytes by the bone marrow. The commercial liver extracts so far available are far from being pure preparations of the active principle and thus can have the action of more than one substance.

6. Effect of the Reactive State of the Bone Marrow

The discussion so far has been based on the results obtained in the average patient with pernicious anemia. In addition to a consideration of the foregoing factors, it is necessary to recognize that certain patients are more or less able than the average subject to develop a reticulocyte response. Thus patients whose initial level of reticulocytes is in the vicinity of 5 to 8 instead of the usual 1 to 3 per cent may be expected to respond more readily but to give less marked increases at the peak. In general, older persons and those with arteriosclerosis respond less readily than younger ones. Extensive neurological involvement is often associated with slightly lower reticulocyte responses. Severe damage to vital organs, such as the liver, is prone to inhibit reticulocyte responses and blood production. By analogy with other anemias it is clear that toxic factors, such as may be associated with nitrogen retention, may act in a similar way. Infection of a variety of types may greatly inhibit blood regeneration. Significantly altered metabolism may perhaps have a similar effect. Difficulty with the absorption of material from the alimentary tract, although not strictly concerned with the reactive stage of the bone marrow, may, as discussed above, be responsible for lessened reticulocyte responses and slow blood production.

7. Effect on Other Blood Elements

Coincident with the increased outpouring of red blood cells from the bone marrow there occurs an increase in the numbers of white blood cells and blood platelets, the former reaching normal or higher than normal values at about the same time as the peak of reticulocytes, and the latter a few days later. Venous thromboses occasionally occur coincident with the response of the three formed bone marrow elements.

At the same time that outpouring of all cellular elements occurs, the plasma bilirubin content, which almost always has been elevated, falls to a normal level. It has been known for many years that there is an increased excretion of bile pigments in the urine and stools of patients with pernicious anemia in relapse. This has often been explained as dependent on increased blood destruction. That some increased blood destruction occurs in this disease can not be denied. However, with the red cells in the vicinity of 1,000,000/ cu. mm., the total pigment excretion is often such that if it be assumed that these pigments are largely derived from blood destruction, this must be progressing at a rate as high as 15 times normal. Such a state of affairs is hardly compatible with life unless there is concomitantly increased blood formation. Although bilirubin is known to be a breakdown product of hemoglobin, it need not be supposed that it is quantitatively derived in the patient with pernicious anemia from the hemoglobin of adult erythrocytes. It is distinctly possible that in the presence of the deficiency which leads to this disease there is a disordered metabolism of pigments, with an increased excretion due to processes which do not derive their raw materials from destroyed red blood cells.

Dobriner and Rhoads (6) have found an increased excretion of coproporphyrin I in three patients with pernicious anemia in relapse and a return to normal values following specific treatment with liver extract. This porphyrin serves no known function in the body and is believed by them to be a by-product in the synthesis of type III protoporphyrin which is found combined with iron and protein in hemoglobin and myoglobin. Its increased excretion in pernicious anemia in relapse is therefore interpreted by them to indicate increased hemoglobin (and erythrocyte) formation The anemia, they believe, is hence due not to decreased blood formation but to increased blood destruction, and they point out the well-known increased excretion in this disease of urobilinogen, which is a breakdown product of coproporphyrin III. They point out that in definitely hemolytic anemias there is a similar increase in the excretion of coproporphyrin I and urobilinogen. Two questions, however, must be answered before it may be concluded from the above that pernicious anemia is essentially due to increased blood destruction. First, does the increased urobilinogen excretion in this disorder owe its origin to increased blood destruction or may it represent the excretion of a product derived from hemoglobin precursors? Second, may not the abnormal pigment metabolism noted by Dobriner and Rhoads result from alterations in the synthesis and breakdown of myoglobin?

III. The Effect of the Anti-Pernicious Anemia Material on Other Manifestations of the Disease

Before discussing other effects of the anti-pernicious anemia material, it is to be emphasized that as used in this section, the term "anti-pernicious anemia material" refers to the substances derived from liver and other organs ordinarily used in the treatment of pernicious anemia. Whether the lack of material which produces certain general manifestations is the same as that which produces the alterations in the blood is not known. It is likewise not known whether the materials effective against the neural and gastro-intestinal manifestations are the same as those producing hematologic effects.

Within 48 to 72 hours after the administration of adequate amounts of the anti-pernicious material, general effects become manifest. The patient feels better, becomes more alert and interested in his surroundings. Appetite improves. Nausea and vomiting cease. Diarrhoea stops. Glossitis begins to cease. No redness or soreness of the tongue is noted after 4 or 5 days. Weeks or months, however, are required before the full regrowth of lingual papillae is complete. In the adequately treated patient this invariably occurs and the tongue eventually appears entirely normal. The repair, with therapy, of lesions of the stomach, is in some cases comparable to that which occurs in the tongue (7, 8), although gastric anacidity, often a precursor of the disease, is rarely *if ever* affected by any type of therapy. In most cases of pernicious anemia, however. striking repair of the gastric mucosa does not result from treatment (9).

Peripheral neuritic signs and symptoms commence to improve in a fortnight. Complete disappearance of these manifestations may be expected. On the other hand, it is well known that regeneration of completely destroyed nerve cells and fibers within the central nervous system does not occur no matter what the nature of the original injury is. Therefore, neural signs resulting from complete destruction of cells and fibers within the spinal cord can not be expected to recede. However, there is reason to believe that before complete degeneration occurs there is for a short period of time a state in which there is physiologic loss of function of a reversible type. Davison (10) states that: "by the early administration of liver extract parenterally, cessation and reduction in the swelling of myelin sheaths can be accomplished, thus preventing further destruction of the axis-cylinders. The function of the axis-cylinders can thus be restored, provided they are not completely destroyed." When the material from liver is given in sufficient amount one very often observes that there is actual regression of spinal cord signs, especially such as are not of long duration, undoubtedly due to the recovery of function of such incompletely degenerated cells and fibers. Furthermore, provided renewed injury is prevented, much improvement may be expected over a long period of time through re-education and by physical therapeutic measures and because it is probable that unaffected cells and fibers may take over functions previously mediated by destroyed pathways.

It is well known that sepsis has a deleterious effect on many kinds of spinal cord lesions and may inhibit the effect of liver therapy not only on the spinal cord but also on the blood in pernicious anemia.

Further, it must be remembered that the amount of anti-pernicious anemia material sufficient to maintain the blood at a normal level may be entirely inadequate for the maintenance of spinal cord integrity. It has been shown that in some cases spinal cord lesions progress in spite of the daily administration of the maximum amount of anti-pernicious anemia material that can be taken within reason by mouth. With the parenteral route there can be administered with ease a vastly larger quantity, and it has been conclusively demonstrated that when enough material is given further degeneration of the spinal cord never occurs (11, 12). It likewise may be noted that patients with pernicious anemia without neural lesions never develop such lesions when receiving adequate therapy (11), although it is known that at least 80 per cent of such individuals would develop neural lesions if untreated.

IV. Effects of Anti-Pernicious Anemia Material on Normal Man and Animals

Liver extracts do not produce polycythemia, but a transient leukocytosis and increase of platelets may follow liver extract injections in normal man or animals. Beneficial results have been reported in some cases of malignant neutropenia following the injection of liver extract. Whether these positive effects depend on precisely the same material effective in restoring the red blood cells to normal in pernicious anemia is unknown. It must not be forgotten that even the more highly concentrated liver extracts are mixtures of substances.

In the grain-fed pigeon the administration of both crude and relatively pure substances effective in pernicious anemia cause a reticulocyte response (13), but a few substances, such as leucine, not effective alone in pernicious anemia, also cause a response (14).

Adult white rats fed an artificial standard diet have been reported to show no changes in the peripheral blood following the administration of liver extract (15). When such animals are kept on a milk diet, however, increases in blood values are said to occur following liver extract administration (16). Thirty to seventy per cent of guinea pigs maintained on a diet of oats, carrots and lettuce and kept in a standard and fixed environment exhibit a reticulocytosis following the administration of material derived from liver effective in pernicious anemia (17).

Other changes in the blood of animals have also been noted. It has been reported (18) but not confirmed (5, 19) that the blood island maturation in early chick blastoderms was accelerated by the addition *in vitro* of liver preparations. It has also been reported (20) and recently denied (21) that the injection of anti-anemia principles alters the blood picture of opossum pouch-young. Accelerated maturation has been observed in the blood of new-born rats whose mothers have been treated with anti-pernicious anemia material (22). This has not been confirmed (21).

Various other positive results with crude liver extract on biological phenomena have been described, such as an acceleration of growth of mosquito larvae (23), but there is no evidence that this is due to the material that affects blood values in pernicious anemia.

Allergic reactions to the injection of liver extract have not been uncommon (24, 25). In some cases the absence of reactions when another brand of potent liver extract was employed, or when extracts derived from the livers of different animals than the one originally causing reactions were employed, have made it evident that it was not the anti-pernicious anemia material itself which was responsible. In other cases, however, the untoward reactions have occurred with any type of liver extract, suggesting either sensitivity to the anti-pernicious anemia material itself or specific sensitivity to any extract derived from liver. Patients sensitive to liver extracts may develop urticarial or shock-like reactions. Desensitization can usually be accomplished by starting with minute injections and increasing the amount daily until the full dose is tolerated. In such individuals it is wise not to allow a greater interval than two weeks between subsequent injection. If desensitization proves to be an unduly difficult procedure in a given patient, it may be feasible to maintain such an individual on oral therapy.

V. Effect of Anti-Pernicious Anemia Material in Disorders of Man Other Than Pernicious Anemia

Beneficial effects on blood formation have been observed in certain macrocytic anemias other than pernicious anemia following liver extract administration. The macrocytic anemia of sprue, "pernicious anemia of pregnancy," tropical macrocytic anemia, and the macrocytic anemia sometimes encountered in patients with intestinal short-circuits or stenoses are among the conditions that have been relieved by the administration of liver extracts. This is presumably due to the fact that all of these macrocytic anemias are closely related etiologically to pernicious anemia, inasmuch as they all result from a lack of specific material derived from food, although the precise nature and mechanism of the deficiency state, as referred to below, may differ from that of classical pernicious anemia.

The fact that relatively crude liver extracts have obviously beneficial effects on the gastro-intestinal and neural manifestations of pernicious anemia has led to their use in various conditions with comparable manifestations. Such crude extracts of liver contain, in addition to the anti-pernicious anemia material, many other substances, including thiamin, nicotinic acid and probably other members of the B group of vitamins. It is therefore obvious that the administration of sufficient quantities of crude liver extracts will be of benefit in pellagra, nutritional polyneuritis and allied conditions. It has also been found that the glossitis present in certain patients with disorders of the liver and pancreas, as well as in those with lesions of the intestine, is benefited by parenterally administered liver extract.

VI. THE FORMATION OF THE ANTI-PERNICIOUS ANEMIA MATERIAL

Castle and his associates (26, 27) have demonstrated that the gastric secretion of patients with classical pernicious anemia is deficient in an essential thermolabile constituent ("intrinsic factor"). The gastric secretion of the normal individual when fed to patients with pernicious anemia was shown to be ineffective in permitting blood regeneration. The daily feeding of 200 g. of beef muscle ("extrinsic factor") had no significant effect. However, when both beefsteak and normal gastric juice are given together, increased blood production and clinical improvement similar to that induced by liver is observed. It is, therefore, probable that Addisonian pernicious anemia usually develops in those individuals whose gastric juice does not contain enough intrinsic factor to react effectively with a factor contained in beefsteak and other foods (see below). Conversely, it is supposed that this gastric factor reacts in the normal individual with a food factor which eventually leads to the production of the active principle or principles which are found in the liver, kidney, brain, placenta, and other organs of normal man and animals. The fact that the methods of extracting potent material from liver fail to give potent extracts of kidney may mean either that the principle is in a different form in the kidney or that other qualities of kidney tissue prevent the extraction. Anti-pernicious anemia material has been found in the livers of various mammals (man, ox, hog, horse, dog, reindeer), of birds, and of fish. Little or none of this material is found in the livers of patients with classical Addisonian pernicious anemia or with the macrocytic anemia of sprue who have died without specific treatment.

Rhoads and Miller (28) have produced achlorhydria, lack of intrinsic factor and macrocytic anemia in swine by feeding diets defective in certain portions of the vitamin B complex. The livers of these experimental animals have been shown by test on patients with pernicious anemia to be deficient in the anti-pernicious anemia material. Bence (29) and Goodman, Geiger, and Claiborn (30) have shown that the livers of gastrectomized swine become progressively depleted of this material, which directly or indirectly permits normal bone marrow function and thus prevents the development of the anemia. On the other hand Wintrobe and others (31) have shown that the feeding of very large amounts of brewer's yeast (45 g. or more daily) to patients with pernicious anemia will bring about remission in about one-third of the cases, suggesting that there may be a small amount of intrinsic factor, present in these patients' gastric secretions, which reacts with a great excess of extrinsic factor to form the anti-pernicious anemia material. This will be discussed more fully below.

Goldhamer (32), in fact, collected 1500 cc. of gastric juice over a period of 8 weeks from 5 untreated patients with pernicious anemia in relapse and then administered 150 cc. of this material daily after incubation with beefsteak to a sixth patient with typical Addisonian pernicious anemia in relapse. A reticulocyte peak value of 9.8 per cent on the 13th day was obtained (red cell level 2.0 million/cu. mm.). In a second experiment he collected 2545 cc. of gastric juice over a period of 12 weeks from 10 patients with pernicious anemia in relapse. This material, similarly administered to a patient with pernicious anemia in relapse led to 16.2 per cent reticulocytes on the 11th day (red cell level 1.0 million/cu. mm.).

Available evidence thus suggests that the interaction of the food and gastric factors proceeds according to the law of mass action, and may schematically be represented by the formula: $\frac{F \times G}{I} = L.\ E.$, in which F stands for the food factor, G for the gastric factor, I for the intestinal impermeability, and $L.E.$ for the final heat-stable factor effective in pernicious anemia. Probably in most cases of macrocytic anemia produced by alteration in this mechanism, none of the factors on the left of the equation is completely normal, and there is a variable participation of defects of one or both of the factors in the numerator or some increase of the denominator value. Any or all of such changes from the normal, if sufficiently great, will create a decrease in the body of the anti-pernicious anemia material. It is now recognized that one may observe this decrease, with consequent macrocytic anemia, from a deficiency of the food factor alone, as in certain cases of sprue, tropical macrocytic anemia, and cases seen in temperate climates. In the usual case of classical pernicious anemia the defect is chiefly in the gastric factor, while in pernicious anemia of pregnancy and many cases of sprue, defects in both gastric and food factors occur. In macrocytic anemia associated with intestinal shunts and stenoses, it is believed that alteration of intestinal absorption plays a rôle. An unknown toxic product might develop under these circumstances and act to destroy or inhibit the action of "liver extract." The probability of a disturbance of the internal metabolism of the anti-pernicious anemia principle has also been recognized.

Following the discovery by Sturgis and Isaacs (33) and by Sharpe (34) of the effectiveness of pig stomach in the treatment of pernicious anemia, the active principle of this organ was found to have a thermolability similar to that of the intrinsic factor of human gastric juice and to differ from that of liver. The effective agent in liver will resist boiling, while the intrinsic gastric juice factor and the effective material in pig stomach is rapidly destroyed by a temperature above 70°C., and more slowly at a temperature as low as 40°C. It is probable that the effectiveness of gastric tissue is dependent on the effect of intrinsic factor acting on extrinsic factor. Meulengracht (35, 36) has shown that the activity of pig stomach is largely confined to the pyloric region; moreover, in that animal at least, active material is found also in the upper portion of the duodenum, where glands similar in structure to those of the pyloric region are present. However both Meulengracht (37) and Magnus and Ungley (38) have pointed out that the degenerative process in the stomach in pernicious anemia does not significantly involve the "pyloric gland organ," inferred by Meulengracht to be the chief source of intrinsic factor in man. Fox and Castle (39) have confirmed and elaborated Meulengracht's observations on the hog stomach. They were unable to develop an active preparation of the fundus by measures designed: (*a*) to prevent the local destruction of intrinsic factor; (*b*) to increase its secretion; (*c*) to activate a zymogen form; or (*d*) to potentiate intrinsic factor by contact with substances in the pylorus region of the stomach. However, when they turned to the human stomach they found those areas containing the *fundus* type of gland and not the "pyloric gland organ" to be of highest anti-pernicious anemia activity. Accordingly in man, the source of intrinsic factor appears to coincide with the site of the degenerative process as seen in histologic preparations of the stomach in pernicious anemia. Recently, effective material has been demonstrated in the lower portions of the small intestine and the colon (40). Observations of Dexter, Heinle, Fox, and Castle (41), however, indicate that, if thoroughly washed, the lower half of the small intestine, in contrast to the stomach and duodenum, is inert, suggesting that the activity of this portion of the bowel results from the absorption of the mixture of the food factor and the intrinsic factor which has been secreted in the stomach and duodenum. However, it must not be assumed that any of the observations on the hog are necessarily applicable to man.

The intrinsic factor has not been identified with any known constituent of the normal gastric secretion. It has been shown not to be hydrochloric acid, pepsin, rennin or lipase. It is present in the gastric secretions of certain patients with gastric achlorhydria, who either have no anemia or have other types of anemia than pernicious anemia. It may be absent from the gastric secretion in certain unusual cases of macrocytic anemia in the presence of normal amounts of hydrochloric acid and of pepsin and rennin. The intrinsic factor is not present in normal human saliva, nor in normal human duodenal secretion from which gastric juice has been excluded.

Evidence for an *in vitro* activity of the intrinsic factor is as yet incomplete. Taylor and his associates (42, 43), however, have shown that normal human gastric juice contains a proteolytic enzyme capable of hydrolyzing casein to the proteose stage in an alkaline medium. This enzyme is differentiated from pepsin by virtue of its inactivity at hydrogen ion concentrations below pH 4.0, and from trypsin by the fact that it does not produce large amounts of amino-nitrogen within 24 hours. Although not proving that this enzyme is identical with the intrinsic factor, the observations that the enzyme activity is retained or destroyed under conditions of temperature and acidity which retain or destroy intrinsic factor activity is of interest. Furthermore, it may be completely removed from gastric juice by adsorption with Lloyd's reagent at pH of 7.4 as may intrinsic factor. Like intrinsic factor it is unable to penetrate certain semi-permeable membranes. In patients with pernicious anemia such enzyme activity is minimal or absent, an observation also made independently by Lasch (44). However, the usual presence of interfering enzymes from the intestine made the *in vitro* method an unsatisfactory one for determining, in cases of pernicious anemia, the amount of proteolysis ascribable to the proteolytic agent of normal human gastric juice.

The extrinsic factor also has not been identified. It is present in beef muscle, egg white, milk, casein, tomato juice, spleen, autolyzed and autoclaved yeast, rice polishings, and wheat germ. Reimann has demonstrated it in liver (45), and Fouts, Helmer, and Zerfas (46) have found the liver fraction "G" of Cohn, Minot and their associates (55) to be an efficient source of extrinsic factor. Hydrolysis of this liver extract with dilute sulfuric acid until its active principle for pernicious anemia has been destroyed does not destroy the extrinsic factor.

The precise conditions necessary for the interaction of the food and gastric factors in the normal individual and their site of interaction are also unknown. However, an effect is obtained when these two substances are administered to patients with pernicious anemia at approximately the same time and may even occur when they are given as much as six hours apart. No effect will be obtained unless their mixture is brought to a pH between

5 and 7. Incubation *in vitro* is not essential to produce a reaction. Heating to 70° to 80°C. for half an hour or boiling for 5 minutes invariably destroys the activity of mixtures of extrinsic and intrinsic factor even after incubation with gastric and duodenal contents. It is thus impossible to find in incubated mixtures of the food and gastric factors any hematopoietic substance with a thermal identity with the active principle of liver. Klein and Wilkinson (47) have reported a slight increase of thermostability by incubation *in vitro* of beef muscle and extracts of the active principle of hog stomach.

Strauss and Castle (48) found that small doses of autolyzed yeast were without effect on patients with pernicious anemia although Wills had shown that they produced improvement in the blood in tropical macrocytic anemia. However, if these small doses of yeast were incubated with normal human gastric juice, typical positive effects on the blood were obtained in pernicious anemia. This they explained by assuming that those cases responding to small amounts of autolyzed yeast alone had intrinsic factor present in the gastric juice. Since then Goodall (49), Ungley (50), and Wintrobe (31), have each independently shown that the daily feeding of large amounts of brewer's yeast (60–120 g.) alone may produce typical positive effects in about one in three patients with pernicious anemia. Ungley and James (51) have further found that alcoholic extracts of yeast, potent when orally administered, are inert upon injection. This fact suggests that yeast in large amounts is effective according to the law of mass action by virtue of small amounts of intrinsic factor remaining in the gastric and intestinal secretions of patients with pernicious anemia. One alternative explanation would be that yeast contains small amounts of pre-formed material effective in pernicious anemia; if this be the case, however, it should be effective on parenteral administration. A third possibility is suggested by Wills' and Evans' work. They have demonstrated that certain relatively pure extracts of liver, in sharp contrast to such liver extracts as "fraction G," although maximally effective in forming blood in pernicious anemia are entirely inert in tropical macrocytic anemia and in a macrocytic anemia experimentally produced in monkeys by the feeding of deficient diets. This indicates that there are etiological differences between the exact deficiency in classical pernicious anemia and certain other macrocytic anemias due to nutritional deficiency.

The etiological significance of the observations of Morris and his associates (52, 53), based upon the injection into patients with pernicious anemia of concentrated normal human gastric juice, is not at present established. The reticulocyte responses observed differed greatly in character from those following the administration of liver or stomach preparations, and improvement of the red cell and hemoglobin level was often delayed. The responses recorded may be of a non-specific nature, like those following the injection of protein derivatives and congo red or the oral administration of potassium arsenite. The careful work of Fouts, Helmer, and Zerfas (54) had made it clear that the process of concentration of gastric juice is essential to the effect upon reticulocyte production. Whether an interaction of intrinsic and extrinsic factors in the gastric juice occurs during concentration or later at the site of injection remains to be learned. That the response is due solely to the action of a gastric "hormone" without interaction with extrinsic factor, as claimed by Morris and co-workers, appears to be unlikely.

VII. CONCLUDING REMARKS

There is no question but that the disease described by Addison in 1849 and 1855 and named by Biermer in 1872 "progressive pernicious anemia" is a nutritional deficiency disorder conditioned by the state of the gastric secretion. It is likewise evident that the same clinical syndrome as well as similar syndromes, given the same or different names, may be dependent upon a lack in the diet or upon faulty absorption or utilization of one or more related dietary factors.

In the future, chemical exploration combined with observations at the bedside will fill many gaps in our knowledge concerning the physiology of nutritional deficiency. This new information will lead to the further prevention and successful treatment of pernicious anemia and related macrocytic anemias.

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The Intermediate Metabolism of the Sex Hormones

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I. INTRODUCTION

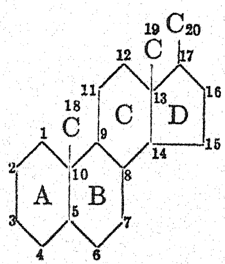
The period from 1929 to 1935 was characterized by the isolation of crystalline substances responsible for the physiological effects produced by materials which can be obtained by the extraction of mammalian sex organs with lipid solvents. The discovery that the hormones were steroidal, *i.e.*, that they contained the perhydro-cyclopenteno-phenanthrene ring system, aroused speculation as to the metabolic relationships which might exist between (a) the sex hormones and cholesterol, (b) the sex hormones *per se*, (c) the sex hormones and the less potent or physiologically inactive steroids of very closely related structure, which for the most part were obtained only incidentally in the isolation of the highly active hormones and which have since become in themselves the object of search. Only in the latter instance (c) has such speculation received any degree of experimental support. Sex hormones in pure form have been administered to human subjects and test animals and the nature of the excretory products studied. The information thereby obtained has been of an indirect nature. More positive information has been recently procured by the isolation of such excretory products in crystalline form. Experiments of this sort were made possible in recent years by a greater availability of crystalline sex hormones. Studies on steroid excretion in patients with hyperplastic or carcinomatous endocrine organs, particularly the adrenal glands, suggest possible pathways in the intermediary metabolism of the steroid hormones, but it is not clear from such studies whether these represent normal or abnormal pathways. As to the site of hormone synthesis, *prima facie* evidence is furnished by the isolation of the steroidal hormones from various endocrine organs of the body; the hormones are in certain instances capable of replacing the organ in many of its sexual functions. Circumstantial evidence regarding the original source of the sex hormones is also procured from studies of steroid excretion and sexual function under various physiological and pathological conditions. Our knowledge of the sites of hormone elaboration is found wanting nonetheless.

Metabolism experiments have been uninformative as to the mechanism whereby the steroid hormones exert their physiological effects. It does not appear at present that the biological transformations which the sex hormones are known to undergo are related to their utilization in the body but rather that these transformations indicate modes of inactivation. Perhaps more extensive investigations involving perfusion of the end organs of sex hormone action, will be more fruitful in this connection; the data are obscured when the administered hormones are permitted to be acted upon by other tissues of the body. Our knowledge of steroid metabolism, meager as it is, furnishes us with some idea as to the immediate precursors of the sex hormones in the animal organism and permits an approach to the more fundamental problem of biosynthesis of the steroid nucleus. The metabolism of the sex hormones in plant microörganisms such as yeasts and bacteria provide useful analogies to that in the animal organism. An enzymatic control of biochemical changes in the steroid molecule is suggested. Indeed, enzyme preparations have been obtained which cause deep-seated changes in the structure of the phenolic estrogens. A more intimate understanding of the catabolism of steroids may give an insight as to the manner in which estrogens promote carcinogenesis; the formation of poly-nuclear aromatic substances akin to the synthetic substances of demonstrated carcinogenic activity has been suggested by Fieser (73).

This article reviews our knowledge of the intermediary metabolism of the estrogens, the male sex hormones, and of progesterone; these hormones belong respectively to the C_{18}, C_{19}, and C_{21} steroid groups. Although it is difficult to discuss the steroid hormones in physiological groups since an overlapping in function has become increasingly apparent, an artificial separation on a structural basis will be found useful. Hormones of the adrenal cortex (C_{21} steroids)[1] will not be discussed except in so far as these are directly related to the metabolism of the androgens.

In order to acquaint the reader not familiar with the system of steroid nomenclature, a brief explanation on this point may be welcome; excessive structural formulation will thereby be avoided. Steroids of the C_{21} series can be reduced to the hydrocarbon pregnane (or allo-pregnane), the skeleton structure of which is represented below. The carbon atoms are numbered in rotation and the four rings are designated as A, B, C, and D.

C_{21}



The configuration of rings A and B in pregnane corresponds to *cis*-decalin, that of the allo-pregnane to *trans*-decalin. By convention, the valence of the hydrogen atom attached to carbon atom 5 is represented by a full line in the first case, whereas in allo-pregnane a broken line is used. Hydrocarbons of the C_{19} series are correspondingly named etio-cholane (*cis*-decalin-type) and etio-allo-cholane or androstane (*trans*-decalin-type).

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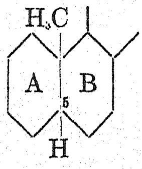
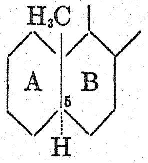
[1] These compounds are dealt with in the review by Reichstein and Shoppee this volume, p. 345.



cis-Decalin-type
C_{21} series: Pregnane
C_{19} series: Etio-cholane



trans-Decalin-type
Allo-pregnane
Etio-allo-cholane or
Androstane

The steric relationship of the hydroxyl group at C_3 to the methyl group attached to C_{10} is indicated by the terms *cis* or β, and *trans* or α. The 3 (β) hydroxylated steroids are usually digitonin-precipitable; the converse does not always follow. The term trans or α applied to the hydroxyl group at C_{17} defines the steric relationship of the functional group to the C_{13}-methyl group. The configuration of the C_{20} hydroxyl group in naturally occurring pregnandiol is arbitrarily referred to as α. The suffixes -ol and -one refer respectively to hydroxyl and carbonyl groups and the point of attachment of these groups is indicated by the number of the carbon atom in the steroid skeleton. The suffix -ene refers to unsaturation and the position of the double bond is indicated by the number following the symbol Δ.

II. PROGESTERONE METABOLISM (C_{21} STEROIDS)

1. Sources of Progesterone (See Table I) and of Progestationally Active Material

a) Corpus Luteum. In 1903, Fraenkel (78) showed that the corpus luteum was essential for the maintenance of pregnancy. In 1928, Corner and Allen (43) prepared active extracts of corpora lutea capable of functionally replacing the organ. This laid the basis for future chemical investigation and at the same time provided a convenient test method for following the fractionation of such extracts. Allen [1932] (2) was responsible for developing methods for the extraction and concentration of hormone preparations from the corpus luteum. In 1934, the isolation of progesterone from this source was announced independently and almost simultaneously from four laboratories (see Table I).

b) Placenta. Progestational activity in extracts of the placenta was demonstrated in 1932 by Mazer and Goldstein (164) and confirmed by many others. More recently activity which may be due to the presence of progesterone has been observed by Smith and Kennard [1937] (204), and by Pincus and Werthessen (180) in extracts of human placentae. This observation has also been confirmed by Fish, Dorfman, and Young [1942] (77). It is generally assumed that the placenta is an important site of progesterone elaboration; indirect evidence for this assumption will be presented in the following discussion. Thus far attempts at the isolation of progesterone from this source have not been reported.

c) Adrenal Glands. For a time it seemed as though the production of progesterone was a function solely of the female organism. However, indirect evidence later pointed to the existence of an additional source. In 1938, Beall isolated the hormone from extracts of ox adrenals (7). This finding has been recently confirmed by von Euw and Reichstein [1941] (72).

TABLE I

The Isolation of Progesterone

Source	When Isolated	Investigator
Corpus luteum	1934	Butenandt (20) Slotta, Ruschig, Fels (202) Allen, Wintersteiner (5) Hartmann, Wettstein (95)
Adrenals	1938 1941	Beall (7) von Euw, Reichstein (72)

2. The Isolation of Products Which Probably Originate from Progesterone (See Table II)

It is interesting that five years prior to the isolation of progesterone from corpus luteum extracts, pregnandiol was obtained by Marrian [1929] (161) from human pregnancy urine; the isolation was incidental to a search for the follicular hormone. This reduction product of progesterone was found to be inactive as a progestin, which is also true of the related steroids subsequently isolated. A number of such metabolites are listed in Table II. Pregnancy urine from human and other mammalian species has proven a particularly rich source of these steroids. The only one of these reduction products of the progestational hormone not isolated exclusively from urine is allo-pregnanolone; the latter substance accompanies progesterone in extracts of the corpus luteum and adrenals. The compounds which are listed may be conveniently classified as ketonic and non-ketonic, alcoholic and non-alcoholic and as digitonin precipitable and digitonin-non-precipitable. Certain of the non-alcoholic ketones of the C_{21} series are slowly precipitated by digitonin although an hydroxyl group is lacking at the C_3 position (27).

The steroids under discussion represent progesterone in various stages of reduction. Pregnandione and allo-pregnandione would thus be derived

TABLE II

The Isolation of Reduction Products of Progesterone

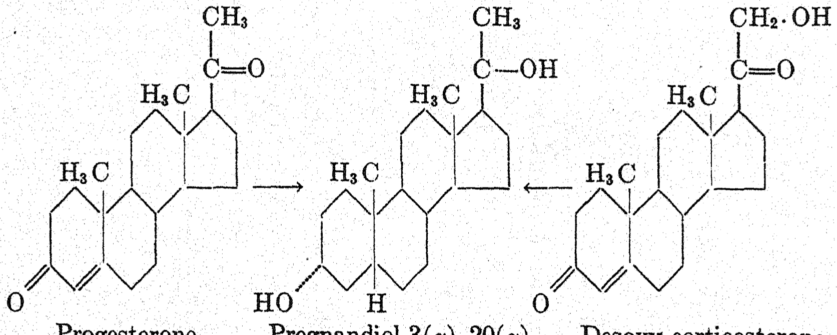
Steroid	Source	Species	Investigator
A. Non-Ketonic: Alcoholic			
1. Digitonin non-precipitable			
Pregnandiol-3(α),20(α) (Pregnandiol)	Pregnancy urine	Man	Marrian [1929] (161)
			Butenandt [1930] (17)
		Chimpanzee	Fish, Dorfman, Young [1942] (77)
		Cow	Marker [1938] (140)
		Mare	Marker, *et al.* [1937] (146)
(Pregnandiol glucuronide)	Non-pregnancy urine	Man	Venning, Browne [1937] (218)
			Marker, *et al.* [1938] (155)
	Non-pregnancy urine (ovariectomized)	Man	Hirschmann [1940] (109)
	Adult male urine	Man	Engel, *et al.* [1941] (68)
		Bull	Marker, *et al.* [1938] (160)
Allopregnandiol-3(α),20-(α)(allo-pregnandiol)	Pregnancy urine	Man	Hartmann, Locher [1935] (94)
		Cow	Marker [1938] (140)
		Mare	Marker, *et al.* [1937] (146)
	Non-pregnancy urine	Man	Marker, *et al.* [1938] (160)
	Adult male urine	Bull	Marker, *et al.* [1938] (160)
Pregnanol-3(α)	Pregnancy urine	Man	Marker, Lawson [1938] (151)
2. Digitonin precipitable			
Allopregnandiol-3(β),20-(α)	Pregnancy urine	Man	Marker, *et al.* [1939] (153)
		Cow	Marker [1938] (140)
		Mare	Marker, Rohrmann [1939] (153)
	Adult male urine	Bull	Marker, *et al.* [1938] (160)

TABLE II—*Concluded*

Steroid	Source	Species	Investigator
B. Ketonic *1. Alcoholic* *a. Digitonin non-precipitable*			
Pregnanol-3(α)-one-20 (epipregnanolone)	Pregnancy urine	Man	Marker, Kamm [1937] (145) Pearlman, Pincus [1942] (174)
		Sow	Marker, Rohrmann [1939] (154)
Allopregnanol-3(α)-one-20 (epiallopregnanolone)	Pregnancy urine	Man	Marker, *et al.* [1937] (148)
b. Digitonin precipitable			
Allopregnanol-3(β)-one-20	Corpus luteum		Butenandt, Westphal [1934] (32) Slotta, *et al.* [1934] (202) Wintersteiner, Allen [1934] (235) Hartmann, Wettstein [1934] (95)
	Adrenals	Beef	Beall [1938] (7)
	Pregnancy urine	Man	Pearlman, *et al.* [1942] (176)
		Mare	Marker, *el al.* [1938] (152) Heard, McKay [1939] (102)
		Sow	Marker, Rohrmann [1939] (154)
2. Non-alcoholic			
Pregnandione-3,20	Pregnancy urine	Mare	Marker, *et al.* [1938] (152)
Allopregnandion-3,20	Pregnancy urine	Mare	Marker, *et al.* [1938] (152)

by the reduction of the double bond in the α,β position to the C_3 carbonyl group in the progesterone molecule. Reduction of the C_3 carbonyl group would yield the corresponding pregnanolones, while reduction of the remaining carbonyl group at C_{20} would give pregnandiol and its stereoisomers. Pregnanol-3(α) is indicative of the high degree at which the reductive processes may operate; the carbonyl group at C_{20} has been completely reduced to a methyl group. Whether Δ^5-pregnendiol is actually derived from progesterone is open to question since a shift of the double bond from the Δ^4 to the Δ^5 position would be required; this question will be discussed in greater detail at a later point. The steps in the reduction of progesterone may not necessarily be in the order described although such a sequence is the simplest.

Of the various products mentioned in Table II, only pregnandiol, *i.e.* pregnandiol-3(α),20(α) has actually been isolated in excessive quantity after the administration of progesterone (Heard, Bauld, and Hoffman) [1941] (99). A pregnandiol complex has been isolated from the urine of men (Buxton and Westphal, 1939) (35), (Hamblen, Cuyler, and Hirst, 1940) (92), and women (Venning and Browne, 1938, 1940) (219, 220), which appears to have been largely derived from the progesterone which had been administered. Failure to isolate other products of progesterone metabolism may perhaps be ascribed to difficulties inherent in the working up of small quantities of material; pregnandiol is comparatively easily isolated because it is difficultly soluble in the usual organic solvents and can be readily extracted from human urines as the glucuronide. It is, of course, possible that the aforementioned C_{21} compounds arise independently of progesterone or are derived from other hormones. Cuyler, Ashley, and Hamblen [1940] (44) were able to isolate considerable amounts of sodium pregnandiol glucuronide from the urine of a man who had been injected with desoxy-corticosterone acetate. Dorfman (private communication) recently administered desoxycorticosterone to a chimpanzee and recovered large amounts of pregnandiol-3(α),20(α) from the hydrolyzed urine. It appears then that pregnandiol may in part arise from progesterone and desoxycorticosterone.



Progesterone Pregnandiol-3(α),20(α) Desoxy-corticosterone

3. Species Differences in Pregnandiol Excretion (see Table III)

It can be seen from Table III that certain quantitative and qualitative differences exist among species in the excretion of pregnandiol and its isomeric diols. In human urines, pregnandiol occurs in conjugation with glucuronic acid (Venning and Browne, 1937) (218), (Venning, 1938) (217). As yet, the pregnandiol glucuronide complex has not been found in the urine of any species other than man. Westphal and Buxton [1939] (233) failed to isolate the complex from the urine of normal and pregnant rabbits, normal and pregnant cats, and from the urine of monkeys injected with progesterone. Sodium pregnandiol glucuronidate could not be found in the urine of guinea pigs to which progesterone and sodium pregnandiol glucuronidate were administered (unpublished observations of Fish, *et al.*). Marker and Hartmann [1940] (144) failed to isolate pregnandiol from hydrolyzed urine of pregnant rhesus monkeys or from the urine of a pregnant rhesus monkey to which progesterone was administered. Elder [1941] (67) was unable to detect the presence of the pregnandiol complex in 24-hour specimens of urine from pregnant chimpanzees. On the other hand, Fish, Dorfman, and Young [1942] (77) have isolated pregnandiol from hydrolyzed urines of pregnant chimpanzees. Heard, Bauld, and Hoffman [1941] (99) found that the pregnandiol which was excreted after the administration of progesterone to rabbits was not conjugated with glucuronic acid. Although Strickler, Walton, and Wilson [1941] (214) were unable to detect sodium pregnandiol glucuronidate in the urine of bulls, large quantities of pregnandiol and its isomers were obtained from hydrolyzed urine (Marker, *et al.*, 1938) (160). It is also interesting to note that while none of the three common pregnandiols could be isolated from sow pregnancy urine, pregnanolones were obtained (Marker and Rohr-

TABLE III

Species Differences in the Urinary Excretion of Pregnandiol and Its Isomers

(Expressed in mg./gal.)

Source and Species	Pregnandiol-3 (α), 20(α)	Allopregnandiol-3(α), 20(α)	Allopregnandiol-3(β), 20(α)
Human pregnancy[1]	50	25	6
Human non-pregnancy[1]	8	4	
Human non-pregnancy (ovariectomized)[2]	0.4		
Chimpanzee pregnancy[3]	3		
Mare pregnancy[1]	50	25	6
Cow pregnancy[1]	25	15	3
Human male[4]	0.2		
Bull[1]	100	50	12

[1] Marker, *et al.* [1938] (160).

[2] Hirschmann [1940] (109).

[3] Fish, Dorfman, Young [1942] (77).

[4] Engel, *et al.* [1941] (68).

mann, 1939) (154). Such differences may be taken to mean that progesterone metabolism does not follow an identical course in all mammalian species.

4. The Relative Importance of Certain Endocrine Organs in Progesterone Production

It has already been mentioned that progesterone has been isolated from the adrenal glands and the corpus luteum; progesterone may very well be the substance responsible for the progestational activity of placental extracts. It might then be asked; what is the extent to which progesterone is elaborated in these organs, and what is the relative order of production? An answer to these questions may be sought by quantitative studies of the excretion of reduction products of progesterone, under physiological and pathological conditions. The assumption is, however, that such products are completely derived from the progestational hormone. Another point that should be kept in mind is that desoxycorticosterone has been shown to give rise to an increased excretion of the pregnandiol complex. The amount of progesterone elaborated by the organism during a given period might also be estimated by the biological effects produced by the hormone, *e.g.* by the extent of proliferation of the uterine endometrium.

Venning and Browne [1937] (218) studied the rate of excretion of the pregnandiol complex during the course of the menstrual cycle. Their data show that the excretion rate may be related to the formation and regression of the corpus luteum. During the secretion phase of the menstrual cycle, 1 to 8 mg. of pregnandiol glucuronide were excreted daily. The peak in the excretion rate was in the latter half of the menstrual cycle; pregnandiol excretion dropped sharply before menstruation (see Fig. 1). It would appear therefore that the corpus luteum is the chief organ of progesterone elaboration in non-pregnant women.

The pregnandiol glucuronidate excretion in pregnant women is markedly increased after the first two months of gestation (Venning, 1938) (217); the excretion rate rises progressively, but falls precipitously at delivery (see Fig. 2). The amount of pregnandiol glucuronide excreted during pregnancy is approximately 10 to 100 mg. daily. The placenta may surpass the corpus luteum in the production of progestational hormone after the second month of gestation and may even become independent of this organ, particularly since removal of the ovaries at later stages of pregnancy does not necessarily lead to the interruption of pregnancy, *e.g.* as in humans, guinea pigs, cats, mares and macacus rhesus monkeys (Robson, 188). Jones and Weil [1938] (117) have obtained the pregnandiol complex from the urine of a woman ovariectomized during pregnancy. A marked rise in the excretion of chorionic gonadotropin was observed to precede the rise in the rate of pregnandiol excretion both during the menstrual cycle (218) and during pregnancy (Browne and Venning, 1936) (15). This might mean that the tissues of the corpus luteum and of the placenta are prepared for the production of progestational hormone. The gonadotropin production may be necessary only for the initiation of this process, since the gonadotropin excretion rate is low at the peak of pregnandiol excretion during pregnancy.

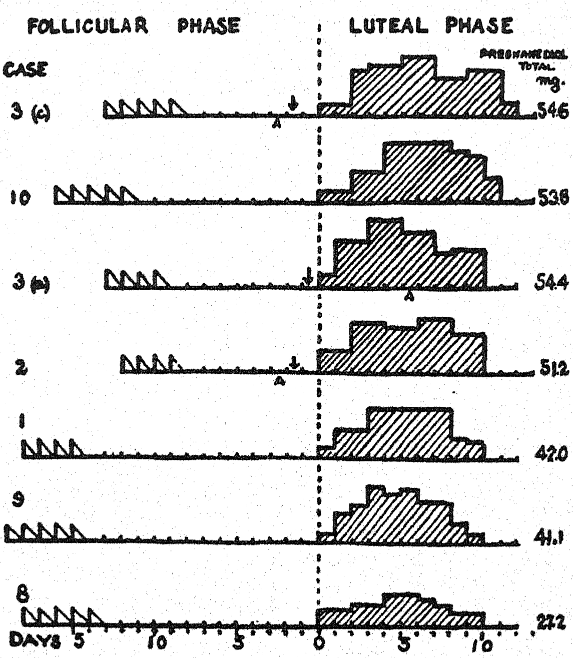


Fig. 1. Excretion of pregnandiol during the menstrual cycle (Venning and Browne) [1937] (218); from Endocrinology

Horizontal lines represent the length of the menstrual cycles; the oblique lines at the beginning of the cycle represent the days of the menstrual bleeding. The shaded part is the excretion of pregnandiol in mg. per day. When gonadotropic substance was present it is marked below the horizontal line by the letter A. Intermenstrual bleeding or intermenstrual pain is denoted by an arrow above the horizontal line.

The amounts of progesterone actually secreted during pregnancy may be approximated by determining how much progesterone must be administered to prevent abortion after ovariectomy. In ovariectomized rabbits, the complete maintenance of pregnancy requires a dosage of 1 mg. per day during the first 10 to 12 days and 2 to 3 mg. per day thereafter (Pincus and Werthessen, 1938) (180) (Allen and Heckel, 1939) (4). In women with toxemia of pregnancy, Smith and Smith [1940, 1941] (207) have reported averting abortion with daily injections of 50 mg. of the hormone, accompanied by estrogen. Evidence of the progesterone requirements of women, however, awaits more elaborate analysis (cf. Hain, 1942) (90).

In humans, the adrenals appear to produce comparatively small amounts of progesterone or desoxycorticosterone if the amount of pregnandiol excreted is to be taken as a criterion. It has already been mentioned that progesterone can be obtained from adrenal extracts and that the hormone may be converted to pregnandiol. Desoxycorticosterone, isolated from adrenals by Reichstein [1938] (186), may also be converted to pregnandiol (44). Engel, *et al.* [1941] (68) found approximately 0.06 mg. of pregnandiol per liter of urine of normal men. Hirschmann [1940] (109) obtained 0.1 mg. per liter from the urine of ovariectomized women; this figure is significantly less than that reported by Venning and Browne [1937] (218) for the urine of normal women. Further evidence that pregnandiol may arise during the metabolism of adrenal cortical compounds has been given by Venning, Weil and Browne [1939] (222) and by Salmon, *et al.* [1941] (193), who isolated sodium pregnandiol glucuronidate from the urine of women with adrenal carcinomata and adrenal hyperplasia, and by Butler

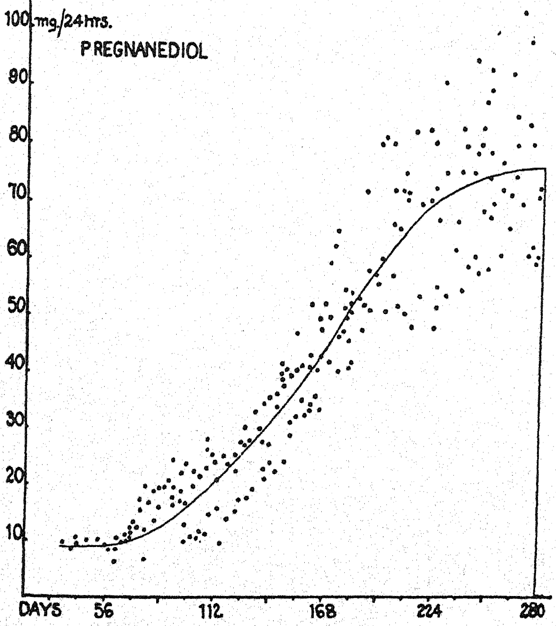


Fig. 2. Urinary excretion of pregnandiol in normal pregnancy (eight cases) (Venning [1938]) (217) (from the Journal of Biological Chemistry)

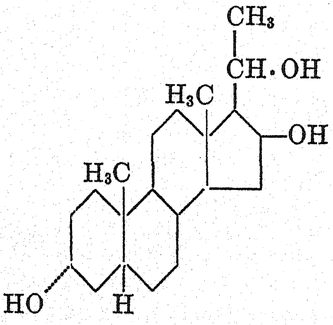
and Marrian [1937] (33) who isolated pregnandiol from the urine of women showing the adreno-genital syndrome.

The testes may in some manner be involved in the formation of pregnandiol, at least in the bull. Marker [1939] (142) has pointed out that while the pregnandiol content of bull's urine is very high, even surpassing that of pregnancy urines, the urine of steers appears to be completely lacking in these steroids. Pregnandiols may be formed in the testes of the bull, or by the influence of the testes on some other gland. Marker also expressed the opinion that while the testes may possibly contain an enzyme capable of reducing the cortical steroids almost quantitatively to the pregnandiols, it is also possible that the bull may utilize a male hormone of 21 carbon atoms which, like progesterone, is reduced to the pregnandiols in the course of its functioning as a hormone.

A theory that a functional uterus is essential for the conversion of progesterone to pregnandiol was proposed by Venning and Browne [1938] (219), by Hamblen, *et al.* [1939] (91) and by others. This theory is no longer tenable in view of the fact that men are capable of converting administered progesterone to pregnandiol (35, 92). Muller [1940] (167) recovered 11.5 mg. of pregnandiol from the urine of an hysterectomized woman who had received 30 mg. of progesterone. Heard, Bauld, and Hoffman [1941] (99) recovered from urine approximately the same quantity of pregnandiol after the administration of progesterone (estrone was simultaneously administered) to adult female rabbits before and after hysterectomy. The yield was 7 to 11 per cent of the progesterone administered. Further data reported by Venning and Browne [1940] (220) indicate that the uterus may normally play a major rôle in the conversion of administered progesterone to pregnandiol but the presence of a normal endometrium is not essential. The site of the conversion of progesterone to pregnandiol in men is not known; Westphal [1940] (232) has suggested the adrenal cortex. It seems that the reduction of progesterone is more concerned with the inactivation of the hormone than with its utilization in causing progestational proliferation. The fact that no progesterone has been isolated from urines which contain large amounts of reduction products of this hormone may be an indication of the rapidity with which progesterone is inactivated biologically.

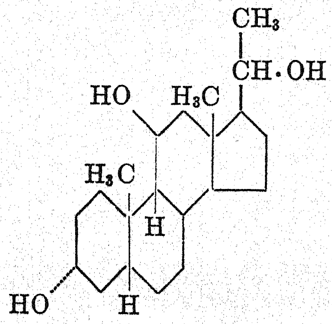
5. *Miscellaneous*

It is highly questionable whether the steroids to be described are derived from progesterone. However, since these compounds have been obtained from pregnancy urine (mare), they may be in some manner related to progesterone metabolism. The first of these compounds is a triol originally isolated from the aforementioned source by Marrian and his co-workers [1933] (203), [1934] (96). It appears to be identical with pregnantriol B isolated from the same source by Marker, *et al.* [1938] (150). Both groups of investigators have assigned positions 3 and 20 to two of the hydroxyl groups. The position of the third hydroxyl group has, however, been more difficult to establish. Marker, *et al.* [1938] (150) at first fixed the position at C_4. Odell and Marrian [1938] (169) showed this to be incorrect and in turn proposed attachment to C_6. Marker and Wittle [1939] (159) next obtained evidence to prove that neither the 4 nor the 6 positions were correct but that the third hydroxyl group was attached to C_{16} of the pregnane skeleton; additional corroboratory evidence was supplied by Marker and Turner [1940] (157). The structural formula for pregnantriol B according to Marker and his co-workers is given below.



Pregnantriol B
or
Allo-pregnantriol-3(α),16,20

Other interesting pregnane compounds isolated from mare's pregnancy urine by Marker and his group are pregnantriol A or urantriol, urandiol-3(β),11, and uranol-11-one-3 (Marker, *et al.*, 1938) (147, 149, 156, 152). It is interesting that these substances possess an oxygen group at C_{11}, a position characteristic of certain adrenal cortical steroids. The urine



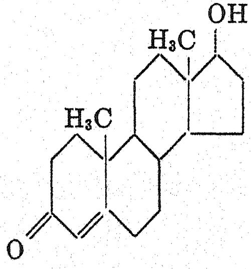
Pregnantriol A or Urantriol

compounds are peculiar in that the hydrogen atom on C_9 has a spatial configuration opposite to that of the naturally occurring steroids. The structural formula for urantriol, according to Marker and his group, is given on page 306.

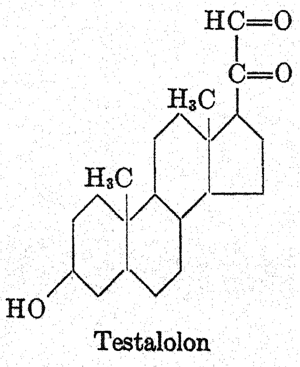
III. Testosterone and Androgen Metabolism (C_{19} Steroids)

1. The Isolation of Testosterone

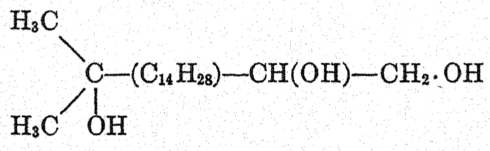
In 1927, McGee (165) working in Koch's laboratory prepared extracts of bull testes capable of promoting comb-growth in capons. At first all attempts to isolate the active hormone from this source were fruitless. With the development of quantitative methods for the bio-assay of androgenic material, a successful attempt at isolation was made by David, Dingemanse, Freud, and Laqueur (47) in 1935. Thus far it appears that testosterone is the characteristic if not the only male hormone of the testes. The structural formula for testosterone is given below.



However, there is some reason for believing that other lipids are present in testis tissue which may be involved in testosterone metabolism. Ogato and Hirano [1934] (170) isolated from hog testes a substance, m.p. 129–130°, which possessed a high degree of activity; the substance, on further characterization, was not identical with androstandione (Hirano, 1936) (107). In a further investigation, Hirano [1936] (108) isolated



Testalolon



$$\mathrm{(H_3C)_2C(OH)-(C_{14}H_{28})-CH(OH)-CH_2\cdot OH}$$

Testriol

four new substances from this source; unfortunately no biological observations were made. One of these compounds, testalolon, is assigned the structural formula given on p. 307. Another compound, testriol, is also described. Another substance is provisionally regarded as the monopalmityl ester of 1,2-propanediol. The remaining compound is not sufficiently well identified to determine its correct empirical formula. Further investigation of testis tissue is highly desirable.

2. The Isolation of Androsterone and Related C_{19} Steroids

In 1928, androgenic activity (Loewe, *et al.*, 122) was demonstrated in extracts of normal male urine. Three years later, the first male hormone, androsterone, was isolated from this source by Butenandt and Tscherning [1931] (29). The capon test, which was placed on a quantitative basis by Gallagher and Koch [1929] (80), was of great value in following the fractionation of the urine extracts. A suspected metabolic relation of androsterone (and certain other steroids later isolated from male urine) (cf. Table IV) to the testicular hormone has recently received experimental support (see Section IV).

That the male gonad is not the only site of androgen elaboration became evident from the observation that a comparable degree of androgenic activity is exhibited by urines of men and women (Koch) [1937–1938] (118). Later, comparable quantities of androsterone and related steroids were actually isolated from normal urines of both sexes (Table IV). The ovaries and the adrenals were then suspected of being directly or indirectly connected with the elaboration of androgenic substances in the female. There is some evidence that the ovaries secrete androgens but the nature of the material is unknown (Hill and Gardner, 1936) (105), (Parkes, 1937) (171), (Deanesley, 1938) (48), (Hill and Strong, 1938) (106). Excessive amounts of isoandrosterone were isolated from the urine of a female exhibiting virilism (Hirschmann, 1941) (110); there was no evidence of adrenal enlargement but the ovaries were polycystic. On the other hand, C_{19} steroids possessing androgenic activity, notably adrenosterone (184) have been obtained from adrenal extracts; other 17-ketosteroids isolated from this source are Δ^4-androstendione, and androstandiol-3(β),11-one-17 (von Euw and Reichstein, 1941) (72).

From Hirschmann's studies [1940] (109) of the urine of ovariectomized women, it appears that the adrenals may be chiefly responsible for the excretion of androgens in women since androsterone and its derivatives were isolated in yields only slightly lower than those reported for normal female urine (Callow and Callow, 1939) (39). The theory that urinary androgens and 17-ketosteroids may be in part derived from the adrenals receives additional support from a study made on the neutral fraction of

TABLE IV

The Isolation of 17-Ketosteroids and Related Steroids: Normal and Pathological Urines

Steroid	Type of Urine	mg./liter	Investigator
A. Ketonic			
1. Alcoholic			
a. Digitonin precipitable			
Δ^5-Androstenol-3(β)-one-17 (dehydro-isoandrosterone)	Men	Qual. detd. 0.2 0.07	Butenandt, Tscherning [1934] (30) Callow, Callow [1940] (40) Engel, *et al.* [1941] (68)
	Men (castrate)	2.0	Callow, Callow [1940] (40)
	Women	0.1	Callow, Callow [1939] (39)
	Women (ovariectomized)	0.1	Hirschmann [1940] (109)
	Girl (adrenal tumor)	110 88	Callow [1936] (37) Wolfe, *et al.* [1941] (238)
	Bull	0.01	Marker [1939] (141)
	Cow (pregnancy)	0.01	Marker [1939] (141)
Androstanol-3(β)-one-(?)	Mare (pregnancy)	Qual. detd.	Heard, McKay [1939] (102)
Etio-allo-cholanol-3(β)-one-17 (isoandrosterone)	Women	0.2	Pearlman [1940] (172) Pearlman [1942] (173)
	Girl (polycystic ovaries)	0.4	Hirschmann [1941] (110)
	Women (adrenal hyperplasia) (unhydrol. urine)	0.5	Butler, Marrian [1938] (34)
	Men (cancer)	0.02	Pearlman [1940] (172) Pearlman [1942] (173)
b. Digitonin non-precipitable			
Etio-allo-cholanol-3(α)-one-17 (androsterone)	Men	Qual. detd. 1.6 0.17	Butenandt, Tscherning, [1931] (29) Callow, Callow [1940] (40) Engel, *et al.* [1941] (68)

TABLE IV—*Continued*

Steroid	Type of Urine	mg./liter	Investigator
A. Ketonic—*Continued*			
1. Alcoholic—Continued			
b. Digitonin non-precipitable—Continued			
Etio-allo-cholanol-3(α)-one-17 (androsterone) —*Continued*	Men (castrate)	0.5	Callow, Callow [1940] (40)
	Women	1.3	Callow, Callow [1939] (39)
	Women (ovariectomized)	1.1	Hirschmann [1940] (109)
	Girl (adrenal tumor)	0.3	Wolfe, *et al.* [1941] (238)
	Pregnant women	Approx. 0.01	Marker, Lawson [1938] (151)
	Bull	Approx. 0.02	Marker [1939] (141)
	Cow (pregnancy)	Approx. 0.02	Marker [1939] (141)
(Androsterone sulfate)	Testicular tumor		Venning, *et al.* [1942] (221)
Etio-cholanol-3(α)-one-17	Men	1.4	Callow, Callow [1940] (40)
		0.03	Engel, *et al.* [1941] (68)
	Women	1.3	Callow, Callow [1939] (39)
	Men (castrate)	0.9	Callow, Callow [1940] (40)
	Girl (adrenal tumor)	13.0	Wolfe, *et al.* [1941] (238)
Androstenol-3(α)-one-17 (double bond position?)	Girl (adrenal tumor)	8	Wolfe, *et al.* [1941] (238)
2. Non-alcoholic			
Androstenone-17	Human urine (normal; adrenal hyperplasia)	Qual. detd.	Dobriner, *et al.* [1942] (54)
	Men (cancer)	0.02	Pearlman [1942] (173)
	Women (ovariectomized)	0.01	Hirschmann [1940] (109)
	Men	Qual. detd.	Engel, *et al.* [1940] (68)

TABLE IV—*Concluded*

Steroid	Type of Urine	mg./liter	Investigator
A. KETONIC—*Continued* *2. Non-alcoholic*—Continued			
$\Delta^{3,5}$-Androstadienone-17	Human urine (normal; adrenal hyperplasia)	Qual. detd.	Dobriner, *et al.* [1942] (54)
	Man (adrenal tumor)	13	Burrows, *et al.* [1937] (16)
	Girl (adrenal tumor)	25	Wolfe, *et al.* [1941] (238)
B. NON-KETONIC			
Etiocholandiol-3(α),17	Men	Qual. detd.	Butenandt [1932] (19) Butenandt, Tscherning, Dannenberg [1937] (31)
Androstentriol-3,16,-17,$C_{19}H_{30}O_3$	A boy (adrenal cortical carcinoma)	>92 mg.	Hirschmann [1942] (111)

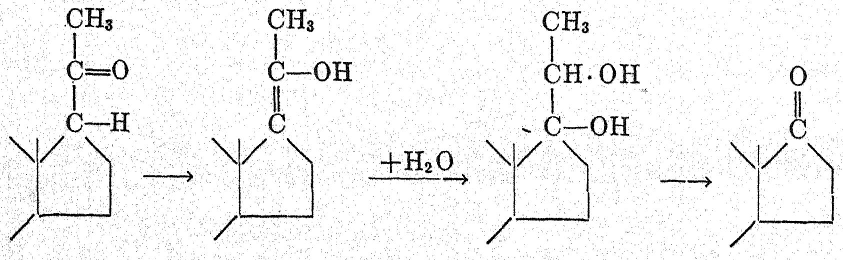
eunuchs' urine by Callow and Callow [1940] (40); considerably more dehydroisoandrosterone but less androsterone and its C_5 epimer were obtained from this source than from normal male urine. Urines obtained from certain cases of adrenal hyperplasia or adrenal tumor yield relatively enormous quantities of dehydroisoandrosterone; a marked increase in the excretion of other 17-ketosteroids of normal occurrence is also observed (cf. Table IV). Dobriner, *et al.* [1942] (54) have given a preliminary report on extensive studies of the 17-ketosteroid content of urines from cases of adrenal cortical pathology; their findings are consistent with those of previous investigators. Urines of this type have yielded 17-ketosteroids not previously obtained from normal urines. Androstenol-3(α)-one-17 was obtained by Wolfe, *et al.* [1941] (238) from the urine of a girl with an adrenal tumor and who exhibited signs of virilism. The double bond in this compound may be at the 6,7-, 7,8-, 9,11- or 11,12-position; the 11,12-position is favored. A highly interesting compound has been recently isolated by Hirschmann [1942] (111) from the urine of a boy with an adrenal cortical tumor. The substance appears to be an androstentriol, $C_{19}H_{30}O_3$; the hydroxyl groups have been assigned to positions 3, 16, and 17 but the configuration of these groups is not established. The position of the double bond is also not known. It has been pointed out that the steroid is structurally analogous to estriol with respect to the position of the hydroxyl groups.

3. Artifacts in the Study of the Intermediary Metabolism of Androgens

Misconceptions of the intermediary metabolism of the neutral steroids may arise from studies based on results obtained from acid-hydrolyzed urines. However, no other recourse than hydrolysis is available since the C_{19} steroids are generally to be found in urines in conjugated form. Mild methods of acid hydrolysis might perhaps be used to advantage in this connection. Certain artifacts are quite obvious. For example, 3-chloro-androstenone-17 was isolated by Butenandt and Dannenberg [1934] (22) from urinary extracts and recognized as having arisen by the action of hydrochloric acid on dehydroisoandrosterone during hydrolysis (Butenandt and Grosse, 1936) (25). The chloroketone has also been obtained by other investigators from urine extracts (238). Androstenone-17 may likewise be an artifact arising from androsterone or from an isomer of this compound. Androsterone sulfate which has been isolated from a pathological urine has been shown (Venning, *et al.*, 1942) (221) to give rise on acid treatment to an androstenone-17 identical with that obtained from normal urines. On the other hand, $\Delta^{3,5}$-androstadienone-17 which has been isolated in large amounts from pathological urines (Burrows, *et al.*, 1937) (16) (Wolfe, Fieser, and Friedgood, 1941) (238) could not be obtained by acid treatment of dehydroisoandrosterone (16). It is possible that the conjugated form in which dehydroisoandrosterone is excreted may be more labile in this respect; Wolfe, *et al.* [1941] (238) are of the opinion that the $\Delta^{3,5}$-dienone may be a transformation product and have suggested Δ^4-dehydroisoandrosterone or its C_3—OH epimer as likely precursors. There may be other excretion products which are likewise sensitive to acid treatment.

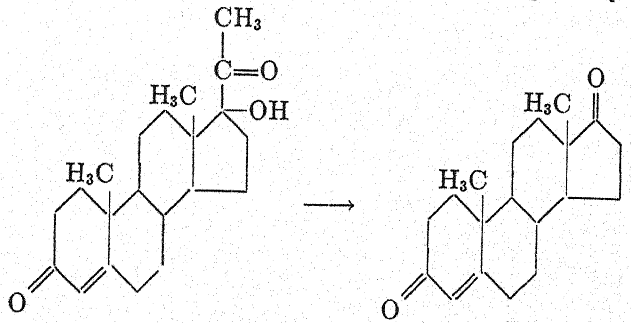
4. Can Progesterone or Other Adrenal Cortical Steroids (C_{21} Steroids) be Converted to 17-Ketosteroids in vivo?

Marrian [1938–1939] (162) suggested that progesterone might be converted into a 17-ketosteroid by the following series of reactions:



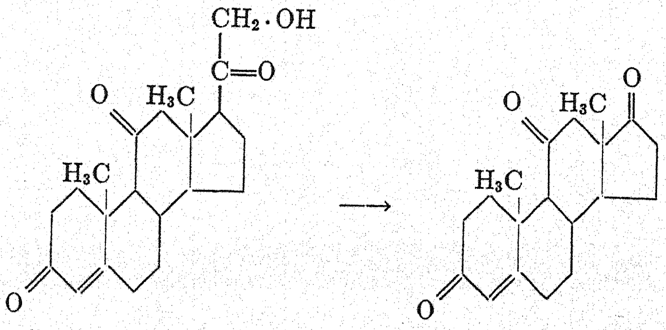
Only ring D of the steroid hormone is represented in the figure, p. 312. This theory offers an explanation for the androgenic activity of the epiallopregnanolone which had been obtained by Marker and his co-workers from pregnancy urine (145, 154). According to the scheme outlined, p. 312, this steroid would be converted, at least partially, into androsterone, which is highly androgenic. However, synthetic epiallopregnanolone fails to exhibit androgenic properties (Heusner, 1938) (104).

It is, nevertheless, conceivable that progesterone may be converted *in vivo* into a 17-ketosteroid which is physiologically inactive, such as etiocholanol-3(α)-one-17. Progesterone has been observed to exert androgenic effects when injected subcutaneously into immature castrated rats but little androgenic activity was noted after intraperitoneal administration (Greene, Burrill, and Thomson, 1940) (87). A marked increase in 17-ketosteroid excretion during pregnancy might be expected in view of the high rate of excretion of the reduction products of progesterone. However, Pearlman and Pincus (unpublished observations) noticed that the 17-ketosteroid values of human pregnancy urine did not differ significantly from those of non-pregnancy. Hain had previously observed [1939] (89) a progressive decrease in the excretion of androgenic substances during the course of human pregnancy. Dorfman and Van Wagenen [1941] (66)



17(β)-Hydroxy-progesterone

Δ⁴-Androstendione



Dehydro-corticosterone

Adrenosterone

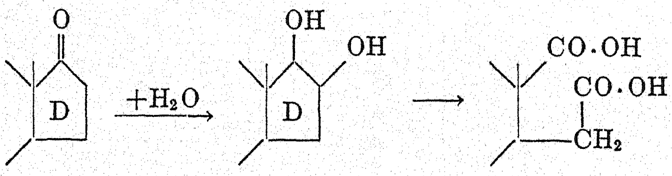
found an increased excretion of androgens into the urine of the rhesus monkey during pregnancy; the increased androgen production was, however, ascribed to the increased activity of the adrenal cortex.

The theory of side-chain oxidation in the intermediary metabolism of adrenal cortical compounds (C_{21} steroids) other than that of progesterone remains an attractive one. Butler and Marrian [1938] (34) have proposed such a theory to account for the occurrence in the urine of women with adrenal cortical pathology of both pregnantriol-3(α),17,20 (33) and etiocholanol-3(α)-one-17 (34). An oxidative removal *in vivo* of the side-chain of the triol to form the latter compound is analogous to that which can be achieved in the laboratory by means of lead tetraacetate. In similar fashion, adrenosterone (184) may be derived *in vivo* from dehydrocorticosterone (184), and Δ^4-androstendione (72) from 17(β)-hydroxyprogesterone (177) (72); all of these compounds have been isolated from adrenal extracts. The high androgenic activity of 17(β)-hydroxyprogesterone (177) is readily explained by a theory of side-chain oxidation.

The 17-ketosteroids which are to be found in urines may arise in part by the metabolism of the adrenal cortical steroids in the fashion indicated above, and the rate of urinary excretion of 17-ketosteroids may therefore be regarded as an index of adrenal cortical activity. The elaboration of testosterone would appear therefore not to be requisite for the formation of 17-ketosteroids.

5. *Theories of Oxidative Scission in vivo of Ring D of the Steroid Molecule*

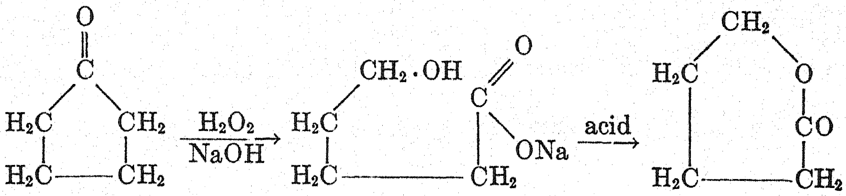
The isolation of a 3,16,17 steroid triol (Hirschmann) [1942] (111) suggests the following series of reactions in the intermediary metabolism of the neutral 17-ketosteroids.



I II III

Step I–II receives a certain degree of support from the recent observation that estrone, a phenolic 17-ketosteroid, is converted *in vivo* to estriol, a 16,17-hydroxylated estrogen (Pearlman and Pincus, 1942) (175), (Pincus and Zahl, 1937) (181). Investigation of the acid fractions of urine extracts may yet reveal the presence of steroids arising as the result of the oxidative process, II→III. In the laboratory, oxidative scission of glycols proceeds smoothly in the presence of lead tetraacetate, especially if the glycol is of the *cis*-configuration.

Another theory of oxidative scission is based on the similarity, emphasized by Dakin [1922] (45), between reactions carried out by the body and those effected through oxidation with hydrogen peroxide. Westerfeld [1942] (228) showed that estrone and cyclopentanone may be converted into lactones by hydrogen peroxide in an alkaline medium (see below); the reaction may prove to be a general one for other cyclic ketones.



Cyclopentanone Lactone

A substance with the empirical formula $C_{19}H_{26}O_3$ which had been isolated from mare's pregnancy urine by Heard [1938] (98) has been recently identified as a ketolactone (Heard and Hoffman, unpublished observations). A closely related ketolactone with the same empirical formula has been isolated by Jacobs and Laqueur [1939] (116).

IV. COMMON FEATURES OF PROGESTERONE AND TESTOSTERONE METABOLISM

Several years ago, Butenandt and his co-workers (Butenandt, Tscherning, and Dannenberg, 1937) (31) drew a comparison between certain urinary products suspected to have arisen during the course of progesterone metabolism and those which might have arisen by the metabolism of testosterone. The comparison has since been extended by Marker and his group as well as by others. Table V lists the steroids to be compared and classifies these according to the structure and configuration of rings A and B in the steroid molecule. The fact that progesterone and testosterone are α,β unsaturated ketones in which rings A and B are identical would lead one to expect similarities in the metabolism of these hormones.

Likely Paths in the Metabolism of Progesterone and Testosterone

Some likely paths of metabolism are indicated in Fig. 3, and have received experimental support from isolation studies in which the hormones under discussion have been administered to human and other species. Thus, progesterone (Type I) is shown to be capable of conversion *in vivo* to pregnandiol-3(α),20(α) (Type VII) (see also comments in Section II, 2). Testosterone is similarly converted to etiocholanol-3(α)-one-17 (Type VII); androsterone (Type IV) and isoandrosterone (Type V) are

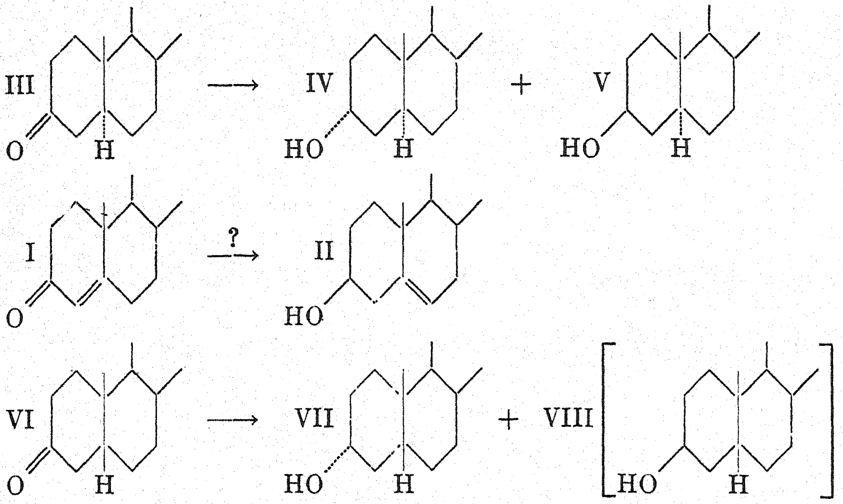


Fig. 3 (Rings A and B only of the steroid molecule are represented)

also formed (Callow, 1939) (38); (Dorfman, Cook, and Hamilton, 1939) (63); (Dorfman and Fish, 1940) (64); (Dorfman, 1940) (61); (Dorfman, 1941) (62). These experiments also show that the hydroxyl group at position 17 can be oxidized *in vivo* to a carbonyl radical. Δ^4-Androstendione (Type I) may also give rise to androsterone (Dorfman and Hamilton, 1940) (65). Hence, testosterone from the testes and Δ^4-androstendione from the adrenal glands, may be reduced in the body to androsterone, a steroid normally occurring in human urines. That Type I steroids may be converted to Type IV by way of Type III is also indicated by the experiments of Dorfman and Hamilton [1940] (65) in which etioallocholandione-3,17 (Type III) was administered to male patients and androsterone (Type IV) isolated in excessive amounts. Since neither Callow nor Dorfman and their co-workers observed an increased excretion of dehydroisoandrosterone (Type II) after testosterone administration, it is questionable whether dehydroisoandrosterone, which is to be found in normal and pathological urines, arises even partially from the testicular hormone. According to Marker [1938] (139) compounds of Type VIII cannot arise in the metabolism of progesterone or testosterone; as yet none have been isolated.

V. Chemical Transformations of Sex Hormones in vitro

1. *General Remarks*

Assuming that the intermediary metabolism of the steroid sex hormones is controlled by enzymatic processes in the gonads, Ercoli, Mamoli, and

Schramm prepared extracts of these organs and found that such extracts were capable of effecting chemical transformations of male hormones (69, 198). It appeared later (cf. Table VII) that the results were largely due to bacterial contamination. Nonetheless, these studies are of great value in providing a method for the study of the metabolism of sex hormones *in vitro*. Although the results thus obtained cannot be strictly compared with those derived from studies of metabolism in the mammalian organism, useful analogies may be drawn.

TABLE V

Some Probable Metabolites of Progesterone and Testosterone

(Isolated from Urinary Sources: See Tables II and IV)

Type (See Fig. 3)	Progesterone (C_{21} Series)	Testosterone(C_{19} Series)
II	a. Δ^5-Pregnendiol-3(β),20(α)	II. b) Δ^5-Androstenol-3(β)-one-17 (dehydro-iso-androsterone)
III	a. Allopregnandione-3,20	III. None isolated
IV	a. Allopregnanol-3(α)-one-20 b. Allopregnandiol-3(α),20(α)	IV. c) Etioallocholanol-3(α)-one-17 (androsterone)
V	a. Allopregnanol-3(β)-one-20 b. Allopregnandiol-3(β),20(α)	V. c) Etioallocholanol-3(β)-one-17 (iso-androsterone)
VI	a. Pregnandione-3,20	VI. None isolated
VII	a. Pregnanol-3(α)-one-20 (epipregnanolone) b. Pregnandiol-3(α),20(α)	VII. c) Etio-cholanol-3(α)-one-17 d) Etio-cholandiol-3(α),17
VIII	None isolated	VIII. None isolated

Numerous metabolism experiments have been carried out in the last few years utilizing yeast and various types of bacteria; the yield of transformation products of the steroid hormones is generally rather high. It is not unlikely that as a result of such studies, isolation of the enzyme systems responsible for the chemical changes may be successfully attempted; this promises to be a fertile field for investigation. Thus far, there have been prepared cell-free extracts and the enzyme systems (of plant origin) isolated, which are capable of inactivating estrogenic hormones. Metabolism of sex hormones does not appear, then, to be confined to specialized endocrine tissues of the mammalian organism. Indeed, synthesis of estrogens occurs in the plant kingdom although the significance of the rôle which these hormones play in plant economy is not known. For example, estrone has been isolated from palm kernel extract (Butenandt and Jacobi, 1933) (26) and estriol from female willow flowers (Skarzynski, 1933) (201); a highly active estrogen has been recently obtained from the Siamese vine (Butenandt, Schoeller, Dohrn, and Hohlweg, 1940) (28).

2. Reactions Involving Rings A and B

a) Male Sex Hormones (C_{19} Steroids). In order to clarify discussion and to avoid excessive formulation, the metabolic products of the neutral steroids have been classified as types (Roman numerals) with respect to the structure of rings A and B as indicated in Fig. 3. Complete identification of the steroid in question may be obtained by consulting Table VI. The reaction processes involving the neutral steroid hormones are summarized in Table VII.

The metabolism of testosterone (Type I) in the animal body as well as in plant organisms indicates that the double bond in α,β position with respect to the carbonyl group attached to position 3, is readily saturated. This may lead to the formation of two types of steroids, (III and VI), both possessing carbonyl groups at position 3 but differing in the spatial configuration of the hydrogen atom at C_5. The reduction of the C_3 carbonyl group also appears to proceed with great rapidity and this apparently accounts for the fact that Types III and VI have not been isolated from animal source material before or after the administration of testosterone to the animal organism. A phytochemical conversion of Type I to Type VI (*e.g.* testosterone to etio-cholanol-17-one-3) has actually been demonstrated. Reduction of the carbonyl group at C_3 may then lead to the production of four new types. Type III would yield Types IV and V; Type VI would yield Types VII and VIII. Metabolism of testosterone (Type I) in the animal body has actually yielded androsterone (Type IV), isoandrosterone (Type V) and etiocholanol-3(α)-one-17 (Type VII), (cf. Section IV). Phytochemical reduction of testosterone has yielded only two of these types (Types V and VII); Type IV (*e.g.* androsterone) has not been obtained. Using an extract of bull testicles, Ercoli [1939] (70) effected the conversion of Δ^4-androstendione-3,17 (Type I) to androsterone (Type IV). A phytochemical reduction of Type III to Type IV (and Type V) has been demonstrated and this sort of reaction can also occur in the animal body, since Dorfman and Hamilton [1940] (65) recovered androsterone (Type IV) after the administration of etioallocholandione-13,17 (Type III). It is interesting that in no instance, neither in the animal body nor in plant organisms, has Type VIII been obtained.

A rule which had been postulated by Mamoli and Vercellone [1937]

(136), [1939] (128) that α,β unsaturated ketones (as of Type I) will not be attacked by fermenting yeast requires some modification. Butenandt and his co-workers (Butenandt, Dannenberg, and Suranyi, 1940) (24) have shown that if the double bond is in a position other than 4,5 but still in conjugation with the carbonyl group at C_3, as in Δ^1-androstendione-3,17, reduction will occur with the formation of isoandrostandiol (Type V).

TABLE VI

(Key to Table VII)

Type	Name of Steroid (C_{21} Series)	Type	Name of Steroid (C_{19} Series)
I. a)	Progesterone	I. c)	Testosterone
I. b)	Desoxycorticosterone	I. d)	Δ^4-Androstendione-3,17
		I. e)	Methyl testosterone
II. a)	Δ^5-Pregnendiol-3(β),20(α)	II. b)	Δ^5-Androstenol-3(β)-one-17 (dehydroisoandrosterone)
b)	(Next column)		
c)	Δ^5-Pregnenol-3(β)-one-20	f)	Δ^5-Androstendiol-3(β),17
d)	Δ^5-Methyl androstenol-3(β)-one-17	g)	Δ^5-Methyl androstendiol-3(β), 17
e)	Δ^5-Pregnendiol-3(β),21(acetate)-one-20		
III. a)	Allopregnandione-3,20	III. b)	Etioallocholandione-3,17
IV. a)	Allopregnanol-3(α)-one-20	IV. c)	Etioallocholanol-3(α)-one-17
b)	Allopregnandiol-3(α),20(α)		
V. a)	Allopregnanol-3(β)-one-20	V. c)	Etioallocholanol-3(β)-one-17 (isoandrosterone)
b)	Allopregnandiol-3(β),20(α)	d)	Androstandiol-3(β),17
VI. a)	Pregnandione-3,20	VI. b)	Etiocholandione-3,17
		c)	Etiocholanol-17-one-3
VII. a)	Pregnanol-3(α)-one-20 (epi-pregnanolone)	VII. c)	Etiocholanol-3(α)-one-17
b)	Pregnandiol-3(α),20(α)	d)	Etiocholandiol-3(α),17

Dehydroisoandrosterone (Type II) has been suggested as a precursor of testosterone in the synthesis of the latter *in vivo*. A phytochemical conversion has indeed been demonstrated; a shift of the double bond in Δ^5-androstendione-3,17 to the 4,5 position has also been shown to occur in the presence of fermenting yeast (Mamoli and Vercellone, 1937) (136),

TABLE VII

Metabolic Reactions of Sex Hormones Involving Rings A and B of the Steroid Molecule; Medium-Yeast, Bacteria

Reactions*	Biological Medium	Investigator
MALE SEX HORMONES (C_{19} SERIES)		
I. c) → VI. c)	"*B. putrefactus*" (yeast water)	Mamoli, Koch, Teschen [1939] (131)
I. c) → VI. c) + VII. d)	Stallion testicle extract (bacterial infection?)	Ercoli [1938] (69)
	"*B. putrefactus*" (yeast brei)	Mamoli, Koch, Teschen [1939] (131)
I. c) → VII. d)	"Putrefactive bacteria" (bull testicle extracts)	Mamoli, Schramm [1938] (133)
I. c) → VII. d) + V. d)	"Putrefactive bacteria" (bull testicle extracts)	Mamoli, Schramm [1938] (132)
I. d) → IV. c)	Bull testicle extract	Ercoli [1939] (70)
I. d) → VII. d) + VI. b)	"Putrefactive bacteria" (bull testicle extracts)	Mamoli, Schramm [1938] (133)
I. d) → VI. b)	Stallion testicle extract (bacterial infection likely)	Ercoli, Mamoli [1938] (71)
	"*B. putrefactus*" (yeast water)	Mamoli, Koch, Teschen [1939] (131)
I. d) → VII. d)	Sow ovaries extract (bacterial infection likely)	Schramm, Mamoli [1938] (198)
I. d) → VI. c) + VII. d)	"*B. putrefactus*" (yeast brei)	Mamoli, Koch, Teschen [1939] (131)
II. b) → I. d)	Yeast (bacterial infection?)	Mamoli, Vercellone [1938] (137)
	Yeast + bacteria	Mamoli, Vercellone [1938] (138)
	Dehydrogenating bacteria	Mamoli [1938] (125)
	Coryne-bacterium mediolanum	Mamoli, Vercellone [1938] (137)

TABLE VII—*Concluded*

Reactions*	Biological Medium	Investigator
MALE SEX HORMONES (C_{19} SERIES)—*Continued*		
II. f) → I. d)	Yeast (bacterial infection?)	Vercellone, Mamoli [1938] (224)
II. g) → I. c)	*Coryne-bact. mediolanum* from yeast + oxygen	Mamoli [1939] (130)
III. b) → V. d)	Fermenting yeast	Vercellone, Mamoli [1937] (223)
III. b) → IV. c) + V. c)	"Putrefactive bacteria" (bull testicle extract)	Mamoli, Schramm [1938] (132)
III. b) → V. c) + V. d)	"*Bacillus putrefactus*" (yeast brei)	Mamoli, Koch, Teschen [1939] (131)
PROGESTERONE, ETC. (C_{21} SERIES)		
I. a) → VI. a)	"*Bacillus putrefactus*" (yeast water or brei)	Mamoli, Koch, Teschen [1939] (131)
II. c) → I. a)	Bacteria (yeast)	Mamoli [1938] (127)
II. e) → I. b)	*Coryne-bact. mediolanum* (yeast water)	Mamoli [1939] (126)

* KEY: Roman Numeral alone refers to configuration in rings A and B of steroid molecule as in Fig. 3; complete identification of the steroid is obtained from Table VI.

resulting in the formation of isoandrostandiol (and testosterone as a by-product). Dorfman and Hamilton [1940] (65) administered dehydroisoandrosterone to male patients but failed to recover crystalline metabolic products from the urine. The reverse process, *i.e.*, the conversion of Type I to Type II has not been demonstrated in either animal or plant metabolism. It seems therefore that dehydroisoandrosterone is not derived, even partially, from the testicular hormone; such a conversion would require a shift of the double bond from the 4,5 to the 5,6 position. This question has been the subject of a polemic between Marker [1941] (143), and Fieser and Wolfe [1941] (74).

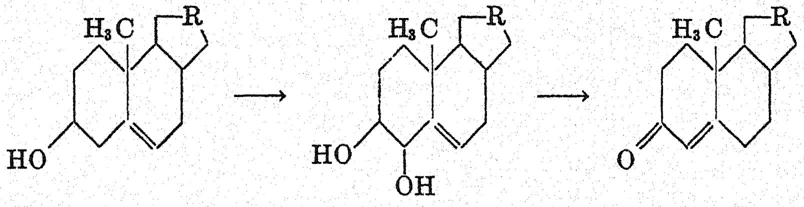
b) *Progesterone* (*C_{21} Steroids*). Similar considerations would apply in this series as in the male hormone series, since both progesterone and testerone are α,β unsaturated ketones. The only metabolite recovered after the administration of progesterone in animal experiments has been pregnandiol-3(α),20(α), (Type VII), although reduction products of progesterone, which include Types II, III, IV, V, VI, and VII have been obtained from various urines (see Section IV). Thus far a phytochemical conversion of Type I to Type VI (progesterone to pregnandione-3,20) has been demonstrated (131), but more extensive investigation may yet indicate other reactions.

Pregnenolone (Type II) has been suggested as a likely precursor of progesterone in the animal body (21). The conversion has actually been accomplished by bacteria (127). The fact that pregnenolone is lacking in progestational activity does not rule out the possibility that the steroid is converted to progesterone in the animal body. This process may occur only in certain tissues of the animal body, *e.g.* the corpus luteum; the process may therefore be obscured by a host of other reactions to which pregnenolone might be subjected before reaching this organ after intramuscular injection. Desoxy-corticosterone (Type I) may also have a precursor of Type II in the animal body; such a conversion has been effected by bacteria (129). The course of the metabolism of rings A and B appears to be in some instances dependent on the nature of the C_{17} side-chain. Cholestenone (Type I) is not attacked by bacteria (Mamoli and Schramm, 1938) (132) (Mamoli, Koch and Teschen, 1939) (131), nor is cholesterol (Type II) (Mamoli, 1938) (127). Fermenting yeast will not reduce cholestanone (Type III) (Mamoli and Vercellone, 1937) (135), nor Δ^1-cholestenone, nor Δ^1-allopregnendione-3,17 (Butenandt, Dannenberg, and Suranyi, 1940) (24).

c) Steroids Other Than the Sex Hormones. It is pertinent to this discussion to include a brief description of a series of experiments in which steroids of non-hormonal nature were fed to mammals and the transformation products isolated from the feces. It is very likely that intestinal bacteria are responsible for the chemical changes which occur (13, 46, 10). Indeed, *Alcaligenes fecalis* can effect oxidative transformations in the case of the bile acids (195, 196).

Schoenheimer and his co-workers (Schoenheimer, Rittenberg, and Graff, 1935) (197) studied possible mechanisms of coprosterol formation from cholesterol in the animal organism. These investigators reported that cholestenone (Type I) when fed to a dog on a basal diet of dog biscuit was converted into cholesterol (Type II), but when added to a meat diet and fed, was converted into coprosterol (Type VIII). It was suggested that the first step in the reaction required the oxidation of cholesterol to cholestenone and the second step, reduction of cholestenone to coprostanone (Type VI). The conversion of coprostanone to coprosterol in a dog and a human was followed with the help of deuterium. The conversion of cholesterol to cholestenone appeared therefore to be a biologically reversible process. Fieser and Wolfe [1941] (74) made the criticism that the cholestenone used in the foregoing experiments carried no indicator element and that this ketone may have stimulated normal sterol excretion, supplanted a normal transformation product of cholesterol or influenced the sterol excretion in some other indirect manner. The experiment could not be considered therefore to have established that cholestenone is capable of undergoing reduction to cholesterol under the influence of intestinal bacteria. Anchel and Schoenheimer [1938] (6) prepared deuterium-containing cholestenone and fed this to dogs. The cholesterol which was subsequently isolated from the feces contained an insignificant amount of deuterium; the conversion of cholestenone to cholesterol appeared therefore to be questionable, but the investigators felt that this process was not ruled out. The conversion of cholestenone to coprosterol could, on the other hand, be followed with the aid of deuterium. In a series of similar experiments, Marker and his co-workers (Marker, Wittbecker, Wagner, and Turner, 1942) (158), confirmed Schoenheimer's findings. Cholestenone (lacking deuterium) was fed to dogs; in addition to the products which have been previously described, epicoprosterol (Type VII) was isolated from the feces. Furthermore, when Δ^4-dihydrotigogenone, a sapogenin of Type I, was fed, reduced sapogenins corresponding to Types II, VII and VIII were isolated. A foundation of analogy sought for by Wolfe, *et al.* (238) for the biological reduction of a Δ^4-3-ketosteroid (Type I) to a Δ^5-3-hydroxysteroid (Type II) has therefore been established in the sapogenin class of steroids.

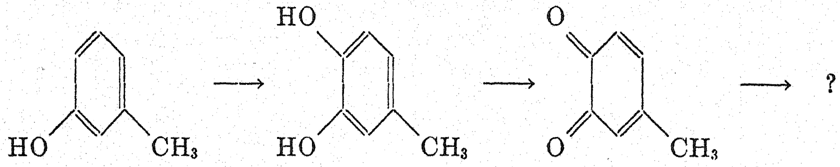
Rosenheim and Starling [1937] (189) have suggested an intermediate step in the hypothetical conversion *in vivo* of cholesterol (Type II) to cholestenone (Type I):



Cholesterol 3,4-Glycol Cholestenone

A 3,4-glycol, obtained from cholesterol by Rosenheim and Starling [1937] (189) with the aid of selenium dioxide, is readily converted to cholestenone by mild acid treatment. The feeding of the cholesterol glycol results in an increased excretion of coprosterol (Rosenheim and Webster, 1935) (190). A substance found in brain tissue facilitates the conversion of cholesterol to coprosterol in animal subjects (Dorée and Gardner, 1938) (60) (Rosenheim and Webster, 1941) (191) and likewise the reduction of β-sitosterol to an analogous product (Rosenheim and Webster, 1941) (192).

d) *Estrogens.* Recent investigations (226, 84, 85, 86, 244) indicate that native estrogens are acted upon by enzymes, causing more or less complete loss in physiological activity. It is very likely that the steroid estrogens undergo oxidative changes in ring A, which is aromatic and possesses a phenolic hydroxyl group. This hypothesis is based on studies of the action of phenolases on simple phenolic compounds (Raper, 1928) (183). The reactions which occur when tyrosinase acts on *m*-cresol, for example, may be indicated as follows:



m-Cresol Homocatechol

An enzymatic conversion of homocatechol to the quinone has been demonstrated; the unstable quinone was obtained in the form of the anilinoderivative. That the reaction normally proceeds beyond this stage is deduced from the observation that, for each molecule of a monohydric phenol, 3 atoms of oxygen are absorbed.

Westerfeld [1940] (226) has presented evidence for the inactivation of estrone by tyrosinase. The oxidative nature of the inactivation process has been demonstrated by Graubard and Pincus in a series of extensive studies (Graubard and Pincus, 1941, 1942) (84, 85, 86). The behavior of the phenolases towards native estrogen parallels their activity towards phenols generally with one notable exception: mushroom tyrosinase does not appreciably oxidize estrogens, nor does it affect their activity. On the other hand, potato tyrosinase oxidizes the estrogens to form colored end-products with the uptake of 3 to 4 atoms of oxygen per molecule of hormone. The hormones appear to be completely inactivated. Laccase oxidizes the estrogens; there is an uptake of about one atom of oxygen per molecule of hormone and a loss of 90 per cent of the hormone activity. The oxidations are inhibited in the presence of cyanide, salicylaldoxime and other copper inhibitors. The estrogens studied were estrone, α-estradiol and estriol. Alcoholic solutions of the steroid hormones were at first used but in later studies water-soluble preparations were employed. Zondek and Sklow [1942] (244) described the preparation of an estrone inactivating enzyme from potatoes, which they believe to be neither a tyrosinase nor a laccase.

It should be emphasized that the aforementioned enzyme preparations have been obtained from plant sources in every instance. Zondek [1934] (242) prepared a liver brei which was capable of inactivating estrogens; the inactivation was ascribed to an "estrinase". The inactivation may have been the result of a detoxification (esterification) process rather than an oxidative one. Graubard and Pincus [1942] (86) were unable to obtain an estrogen oxidase from animal tissues, particularly from the liver. However, Heller [1940] (103) found that hydrolysis did not result in liberation of activity of the estradiol which had been inactivated by incubation with liver slices. Heller therefore suggested that the inactivation might be due to enzymatic destruction rather than to conjugation or esterification. He observed that the addition of cyanide to the liver-estradiol incubation system caused complete inhibition of the inactivating system. Cyanide has no effect on dehydrogenases (there are a few exceptions) but does inhibit oxidases strongly. Various poisons which are known to inhibit dehydrogenases did not prevent destruction of estradiol when these were added to the system. It was suggested, therefore, by this investigator that the inactivation of estrogens by liver (and also kidney) was effected by an oxygenase system. Since cyanide inactivates the liver detoxifying system (121), detoxification is not ruled out as an inactivating process in Heller's experiments. Cadden and Dill [1942] (36) have described some properties of a polyphenol oxidase present in cell-free kidney extracts. It would be interesting to see whether this oxidase will inactivate estrogens.

3. *Reactions Involving Ring D and the C_{17} Side-Chain*

a) Reduction of the C_{17} Carbonyl to an Hydroxyl Group. A phytochemical conversion of this sort has been amply demonstrated. A number of examples are indicated in Table VII and others have been mentioned in the text. Additional examples which might be cited are the following conversions which occur in the presence of fermenting yeast:

(1) Dehydro-iso-androsterone to Δ^5-androstendiol-3,17 (Mamoli and Vercellone, 1937) (134).
(2) Δ^4-Androstendione-3,17 to testosterone (Mamoli and Vercellone, 1937) (135).
(3) Δ^1-Androstendione-3,17 to Δ^1-androstenol-17-one-3 (Butenandt and Dannenberg, 1938) (23).

The conversion of dehydroisoandrosterone to Δ^5-androstendiol-3,17 occurs also in the presence of "*Bacillus putrefactus*"[2] added to sterile yeast brei (Mamoli, Koch, and Teschen, 1939) (131). Estrone, a 17-ketosteroid estrogen, is capable of undergoing similar reduction in the presence of fermenting yeast, with the formation of α-estradiol; little or no β-estradiol is formed (Mamoli, 1938) (126). Wettstein [1939] (234) confirmed these observations but found that it was not necessary for the estrogen to be esterified in order for the reduction to take place. A similar reduction of estrone has been shown to occur also in the animal body (see Section VI).

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[2] Possibly identical with *Clostridium putrefaciens*.

b) Oxidation of the C_{17} Hydroxyl to a Carbonyl Group. Vercellone and Mamoli [1938] (224) found that Δ^5-androstendiol-3,17 was oxidized in the presence of yeast (Mailand strain) to Δ^4-androstendione-3,17; dehydrogenating bacteria may have been responsible for the reaction. As has already been mentioned, testosterone is converted into a number of 17-ketosteroids in the mammalian organism. Dorfman and Hamilton [1940] (65) administered etioallocholandiol-3(α),17 to male patients and recovered an excessive amount of androsterone from the urine. Oxidation of the C_{17} hydroxyl group of estradiol has been shown to occur also in the animal body (see Section V).

c) Oxidations in the C_{17} Side Chain. Oxidations of this sort have not as yet been demonstrated to occur in either plant or animal metabolism. Mamoli [1939] (126) found that the C_{21} hydroxyl group of Δ^5-pregnendiol-3(β),21-one-20 was not attacked by "*Coryne-bacterium mediolanum*" (in yeast water) although oxidation of the C_3 hydroxyl group did occur; the C_{21} hydroxyl group of Δ^5-pregnentriol-17,20,21-one-3 was likewise not attacked. These experiments fail therefore to provide an analogy for the theory of side chain oxidation in the intermediary metabolism *in vivo* of C_{21} steroids of adrenal origin (see Section III, 4).

VI. Estrogen Metabolism

1. *The Isolation of Estrogens (see Table VIII)*

Certain of the physiological effects produced by estrogens serve as a guide in the fractionation of extracts, culminating in the isolation of crystalline estrogens. The vaginal smear test as described by Allen and Doisy [1923] (3) is especially valuable in this regard. Estrogens have been isolated from the following animal organs: ovaries, placenta, adrenals and testes. Estrogens make their appearance in urines, from which source they are more readily isolated; hydrolysis is required to release estrogens from the conjugated forms in which they are chiefly excreted. Although estrone has not been isolated from ovaries, strong evidence has been obtained for the presence of estrone in the follicular fluid of this organ (Westerfeld, Thayer, MacCorquodale, and Doisy, 1935) (231). Species differences may account for the fact that estriol has been isolated from the human placenta and from no other endocrine organ.

TABLE VIII

The Isolation of Estrogens from Animal Sources

Estrogen	Source	Investigator
	A. KETONIC ESTROGENS	
Theelin or estrone, $C_{18}H_{22}O_2$	Pregnancy urine (human)	Doisy, Veler, Thayer [1929] (59) Butenandt [1929] (18)
	(Mares) pregnancy urine	DeJongh, Kober, Laqueur [1931] (49)
	Male urine (human)	Dingemanse, Laqueur, Mühlbock [1938] (52)
	(Stallion) urine	Haussler [1934] (97) Deulofeu, Ferrari [1934] (50)
	Placenta, human	Westerfeld, MacCorquodale, Thayer, Doisy [1938] (230)
	Testes, stallion	Beall [1940] (9)
	Adrenal, beef	Beall [1939] (8)
Estrone sulfate	Pregnancy urine (mares)	Schachter, Marrian [1938] (194)
Equilin, $C_{18}H_{20}O_2$	Pregnancy urine (mares)	Girard, Sandulesco, Fridenson, Rutgers [1932] (82)
Hippulin, $C_{18}H_{20}O_2$	Pregnancy urine (mares)	Girard, Sandulesco, Fridenson, Rutgers [1932] (82)
Equilenin, $C_{18}H_{18}O_2$	Pregnancy urine (mares)	Girard, Sandulesco, Fridenson, Gaudefroy, Rutgers [1932] (81)
	B. NON-KETONIC ESTROGENS	
Dihydrotheelin or α-estradiol, $C_{18}H_{24}O_2$	Pregnancy urine (human)	Smith, Smith, Huffman, MacCorquodale, Thayer, Doisy [1939] (208)
	Pregnancy urine (mares)	Wintersteiner, Schwenk, Whitman [1935] (237)
	Ovaries (sow)	MacCorquodale, Thayer, Doisy [1935] (124)
	Placenta (human)	Huffman, Thayer, Doisy [1940] (115)
	Testes (stallion)	Beall [1940] (9)

TABLE VIII—*Continued*

Estrogen	Source	Investigator
B. NON-KETONIC ESTROGENS—*Continued*		
β-Estradiol, $C_{18}H_{24}O_2$	Pregnancy urine (mares)	Hirschmann, Wintersteiner [1937] (112)
Theelol or Estriol, $C_{18}H_{24}O_3$	Pregnancy urine (human)	Marrian [1930] (163) Doisy, Thayer, Levin, Curtis [1930] (57)
	Placenta (human)	Browne, Collip [1931] (14)
Estriol glucuronide	Pregnancy urine (human)	Cohen, Marrian [1935] (42)
17-Dihydroequilenin, $C_{18}H_{20}O_2$	Pregnancy urine (mares)	Wintersteiner, Schwenk, Hirschmann, Whitman [1936] (236)

2. *Likely Sites of Estrogen Synthesis*

The mere fact that estrogens can be isolated from a particular organ does not necessarily mean that the organ is capable of synthesizing estrogens, but it does establish a foundation for such an hypothesis. Studies of estrogen excretion under various normal and pathological conditions furnish some idea as to the extent to which estrogens may be elaborated in certain endocrine organs. Such studies have, for the most part, been recently reviewed by Doisy, Thayer, and Van Bruggen [1942] (58).

a) *Ovaries.* Removal of the ovaries terminates sexual function and causes atrophy of accessory genital organs in the female. Injection of estrogenic hormones into spayed females induces all the effects characteristic of the follicular phase of the estrous cycle. The rate of estrogen excretion in the urine varies during the stages of the menstrual cycle and appears to be related to the secretory state of the ovaries. Doisy, *et al.* (58) have calculated that the normal woman produces approximately 10 mg. of estrogen per menstrual cycle.

b) *Placenta.* Estrogen excretion rises markedly during the course of pregnancy and drops precipitously with the termination of pregnancy. Removal of the ovaries of pregnant women (182, 88, 1, and others) and of mares (93) does not cause a disappearance of estrogens from the urine. Dorfman and Van Wagenen [1941] (66) found the removal of ovaries and fetus ineffective in altering the excretion rate in pregnant monkeys, but expulsion of the placenta caused a drop to non-pregnant levels. On the basis of excretion rates during normal pregnancy, and the rate of destruction of injected estrogen in non-pregnant women, Doisy, *et al.* (58) calculated that the incredible amount of 150–600 mg. of estrogen would have to be produced daily. They regard it as unlikely that such large quantities are actually produced but rather that the velocity of estrogen catabolism is impeded in the pregnant state. Smith and Smith (private communication) have found a recovery in the urine of 80% of the estriol injected into a pregnant woman. This would indicate a placental production rate of only 12 to 50 mg. of estrogen per day in late pregnancy. It should be noted that estriol is the principal urinary estrogen in human pregnancy.

c) *Adrenals.* Feminizing effects and the excretion of abnormal amounts of estrogens have been observed in males with adrenal tumors (16); loss of the feminization followed successful removal of the tumor (200, 113). DBA strain mice, which were ovariectomized at birth, develop nodular hyperplasia of the adrenals and mammary glands and go through estrous cycles (239). Administration to ovariectomized rats of extracts of the pituitary which produce stimulation of the adrenal cortex causes the establishment of the vaginal introitus (166) and enlargement of the sex accessories (168). Ovariectomized-adrenalectomized rats show greatly delayed opening of the vagina or complete absence of this organ (225). Ovariectomized women excrete estrogens (79).

d) *Testes.* The high estrogen content of the urine of stallions (Zondek, 1933, 1934) (241) may be the result of testicular activity; horse testes appear to be the tissue richest in estrogenic hormone (Zondek) (240), (Beall, 1940) (9). Very small amounts of estrone have been obtained, however, from the urine of men (52). The urinary excretion of estrogens is lowered, but not abolished in hypogonadal males (Koch, 118). To what extent the testes as well as the adrenals contribute to normal estrogen elaboration is not well established.

3. Chemical Reactions of the Estrogens in vivo

The fact that more than one estrogenic hormone occurs naturally, suggests the hypothesis that α-estradiol, the most potent estrogen, may give rise to less potent estrogens; converse reactions might likewise occur. In order to elucidate this problem, pure hormones have been administered to humans and animals, and a search made for the metabolic products. The results are summarized in Table IX. In recent years, estrogenic steroids have become more readily available, permitting the administration of amounts large enough so that active metabolic products can be isolated and identified chemically. In view of the difficulties of isolation, failure to recover certain estrogens does not necessarily imply their absence.

More comprehensive but less positive information can be obtained by comparing the chemical and physical properties of the substances responsible for the estrogenic activity of post-injection urine extracts with those

TABLE IX

Estrogen Metabolism Experiments

Estrogen Metabolites*		Subject	Investigator
Isolated	Indicated†		
A. ADMINISTERED α-ESTRADIOL			
Estrone β-Estradiol		Rabbits; female (with and without simultaneous progesterone administration)	Heard, Bauld, Hoffman [1941] (99)
		Rabbits: ovariect.-hysterect.	Fish, Dorfman [1942] (76)
Estrone	"Strong phenolic" estrogen (estriol?)	Guinea pigs: both sexes; ovariectomized	Fish, Dorfman [1940, 1941] (75)
Estrone		A man	Heard, Hoffman [1941] (101)
	Ketonic estrogen (estrone?)	Monkeys: female; ovariect.; ovariect.-hysterect.	Westerfeld, Doisy [1937] (229)
	Estrone	Rabbits: female; (ovaries essential to conversion)	Pincus, Zahl [1937] (181)
	Estriol	Rabbits: female; (functional uterus essential to conversion)	
	Ketonic estrogen (estrone?)	Dogs: both sexes	Dingemanse, Tyzlowitz [1941] (53)
None		Pregnant monkeys	Marker, Hartman [1940] (144)

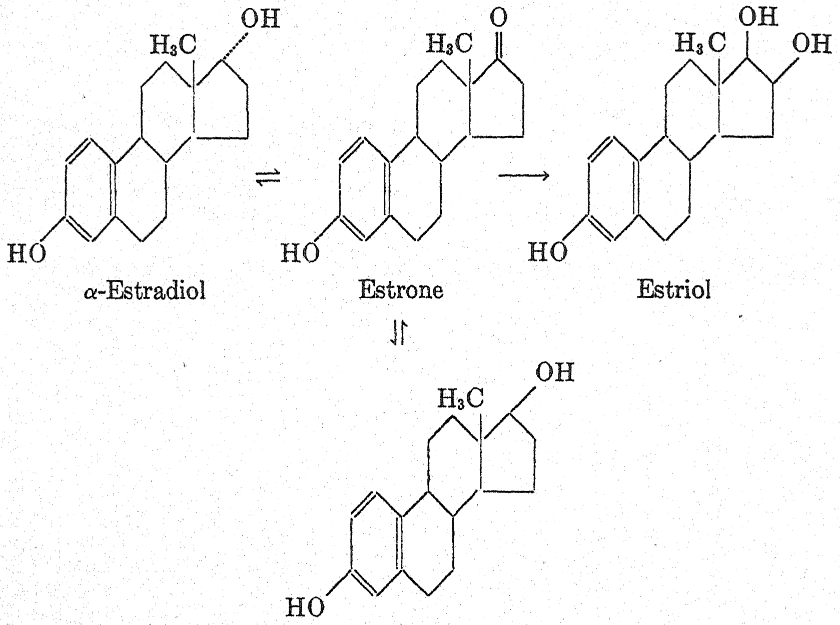
* Excreted in urine except where otherwise noted.

† Indicated indirectly by comparison of the chemical and physical properties of the substances responsible for the estrogenic activity of post-injection urine extracts with those of pure crystalline hormones.

TABLE IX—*Continued*

Estrogen Metabolites*		Subject	Investigator
Isolated	Indicated†		
B. ADMINISTERED ESTRONE			
β-Estradiol		Rabbits	Stroud [1939] (215)
Estriol	α-Estradiol	Men	Pearlman, Pincus [1942] (175)
	Non-ketonic estrogens	Monkeys: female ovariect.; ovariect.-hysterect.	Westerfeld, Doisy [1937] (229)
	Estriol	Rabbit: female; (functional uterus essential to conversion)	Pincus, Zahl [1937] (181)
	Estradiol Estriol	Women (simultaneous progesterone adminis.)	Smith, Smith [1938] (206)
	Non-ketonic estrogens	Dogs: (both sexes)	Dingemanse, Tyzlowitz [1941] (53)
	Non-ketonic estrogens	Dogs: (sex?)	Longwell, McKee [1942] (123)
	In bile: "weak" and "strong" phenolic estrogens		
	Estradiol Estriol	Men and women	Pincus, Pearlman [1942] (179)
	α-Estradiol	Rat and rabbit: liver, uterus, etc. slices	Heller [1940] (103)
C. ADMINISTERED ESTRIOL			
	No estrone, nor estradiol	Monkey: female; normal intact; ovariect.-hysterect.	Doisy, Thayer, Van Bruggen [1942] (58)
		Rabbits: female	Pincus, Zahl [1937] (181)
D. ADMINISTERED β-ESTRADIOL			
	Estrone, α-estradiol?, estriol	Monkey: female; ovariect.-hysterect.	Doisy, Thayer, Van Bruggen [1942] (58)

of pure crystalline hormones. It appears that the following reactions can occur in the mammalian organism.

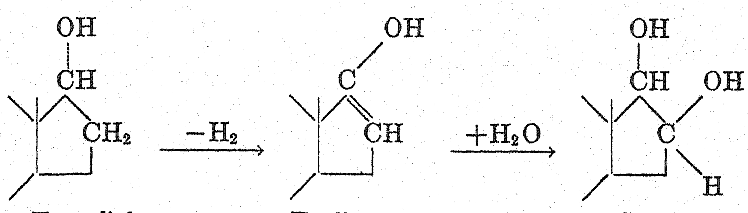


β-Estradiol

In some species certain of these reactions may be favored even to the exclusion of others. Thus, for example, β-estradiol was recovered in relatively large amounts from the urine of rabbits following the administration of α-estradiol (Heard, Bauld, and Hoffman, 1941) (99) (Fish and Dorfman, 1942) (76); β-estradiol but no α-estradiol was recovered after estrone administration (Stroud, 1939) (215). In contrast, α-estradiol but little or no β-estradiol could be detected in the urine of men after estrone administration (Pearlman and Pincus) (unpublished observations).

The bulk of evidence indicates that the ovaries and the uterus are not essential to these transformations; the liver and other non-endocrine organs of the body may effect transformations of estrogens *in vitro* (Heller, 1940) (103). Progestin probably facilitates the conversion of estrone to estriol. This is indicated by the data of Pincus and Zahl (181) who found evidence for the excretion of estriol in rabbits upon the administration of estrone with progesterone. In the luteal phase of the menstrual cycle there appears to be a tendency for increased estriol excretion (209, 206). It is of course well known that estriol is the principle estrogen excreted during human pregnancy when progestin secretion is maximal.

It is not established whether the formation of estrone is a necessary step in the conversion of the isomeric estradiols to estriol. According to Fieser [1937] (73), a transformation of estrone to estriol in the animal organism may require hydration of the enolic form of estrone. Marrian (162) proposed that estradiol is dehydrogenated to yield the enolic form of estrone which is then hydrated to form estriol as follows:



α-Estradiol Enolic form of Estrone Estriol

The occurrence of equilin and equilenin in the urine of pregnant mares suggests that the latter substance is formed by the dehydrogenation of equilin *in vivo*, especially since this reaction readily occurs in the laboratory. Fieser (73) believes that compounds of the equilenin group may represent successive stages in the dehydrogenation of estrone in the Equidae.

4. Estrogen Inactivation

Estrogen metabolism experiments reveal that most of the activity of the estrogens which are administered is lost within a short period of time. Thus, Heard and Hoffman [1941] (101) found that only 10.3 per cent of the α-estradiol which had been injected into a man could be recovered from the urine as phenolic estrogen. In the case of human subjects of both sexes, less than 2 per cent of the injected estrone could be recovered (179). Numerous similar examples could be cited. Only small amounts of injected estrogens are excreted with the feces (Dingemanse and Laqueur, 1937) (51), (Zondek, 1941) (243), (Dohrn and Faure; 1928) (55); (Siebke, 1930) (199) (and others). Cantarow and his co-workers, [1942] (41) have recently obtained evidence for an enterohepatic circulation of estrogens in dogs, a factor which they believe explains the apparent inactivation of estrogens. However, some of their data are in disagreement with those of Longwell and McKee [1942] (123) who observed that only 1.3–8 percent of the administered estrogen was excreted into the bile. Furthermore, much evidence has accumulated which points to the liver as an important site of estrogen inactivation (11, 83, 216, 178).

Smith, Smith, and Pincus [1938] (209) have reported that progesterone retards the processes of estrogen destruction; this may be an important factor in retarding estrogen breakdown in pregnancy. However, Heard, Bauld, and Hoffman [1941] (99) obtained almost equal estrogen recovery in the non-pregnant rabbit whether progesterone was simultaneously injected with estrone or not. Smith and Smith (private communication) recovered less estrogen from the urine of postmenopausal women than from premenopausal women after the administration of the hormone; a diminished production of progesterone in the postmenopausal state may have been responsible.

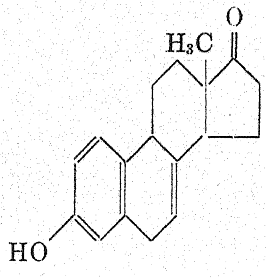
5. *Theories of Estrogen Inactivation*

a) *Formation of Conjugated Estrogens.* Conjugated estrogens of the type that have been isolated from animal sources are almost inactive but liberation of estrogenic activity results on hydrolysis. Studies on estrogen inactivation must, therefore, take this factor into account. Hydrolysis procedures may involve considerable loss of activity according to Doisy [1942] (56), but the data of Smith and Smith [1935] (205) show that not more than 20 per cent of the activity originally present in urine extracts is lost. It is, however, possible that the conjugated forms, in which estrogens are excreted in urines, are more labile than the free steroids which have been subjected to the routine hydrolytic procedures.

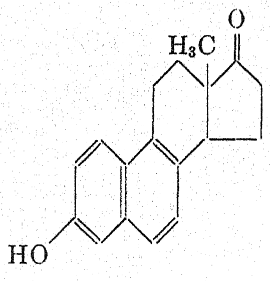
b) *Oxidative Processes.* Ring A of the steroid molecule may be attacked in a manner already described in Section V, Part 2 d. Oxidation in ring D may also occur. Marrian (162) has suggested that estriol may be dehydrogenated *in vivo* to form 16-keto-estrone. Huffman [1942] (114) has successfully converted estrone to this diketone in the laboratory; a 16-hydroxy-estrone (and a compound isomeric with naturally occurring estriol) were also obtained. A study of the biological and chemical properties of these compounds may be useful in the search for new naturally occurring estrogens. Pearlman and Pincus (unpublished observations) have obtained evidence for the presence of an estrogen in human pregnancy urine which is ketonic and contains an hydroxyl group other than the usual phenolic one; 16-hydroxy-estrone is an estrogen of this type although it may not necessarily be the one in question. Smith and Smith [1941] (210) have obtained evidence for the presence of a new compound which may be important in the catabolism of estrogens; this substance is not estrone and can be converted by hydrolysis and the reducing action of acid and zinc into a product of higher estrogenic potency. It is not unlikely that 16-keto-estrone is in turn oxidized *in vivo* to form 16,17-dicarboxylic acids, since one might expect a 16,17-diketone to be very labile. This may be an important path in estrogen catabolism, especially since it has been shown that estrone and the isomeric estradiols are converted in part to estriol *in vivo*. Investigation of the acid fractions of urine extracts may prove fruitful in this regard.

Oxidation of ring D may also result in the formation of lactones (cf. III, pt. 5 for additional discussion). A lactone has been obtained by Westerfeld [1941] (227), [1942] (228) from estrone by the action of H_2O_2. Since oxidations by H_2O_2 have been frequently assumed to resemble *in vivo* oxidative processes, a search for Westerfeld's lactone would be well justified; the lactone would appear in the neutral fraction of urine extracts and not in the acid fraction if the usual fractional procedures are adhered to (101). However, in an estrogen metabolism experiment by Heard and Hoffman [1941] (101), a search for this lactone has been unsuccessful.

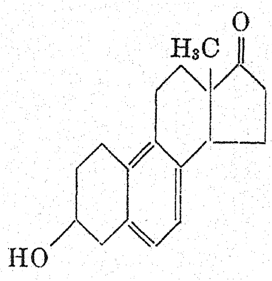
c) *Reductive Processes.* A reduction *in vivo* of ring A of the estrogen molecule may yield the inactive estrandiols which Marker and his coworkers [1938] (155) isolated from human non-pregnancy urine. Marker, *et al.* explain the absence of estrandiols in pregnancy urines and their presence in non-pregnancy urines by a theory of hormone utilization in controlling the estrous cycle. The presence of $\Delta^{5,7,9}$-estratrienol-3-one-17 in the urine of pregnant mares (Heard and Hoffman, 1940, 1941) (100) may indicate a reduction of ring A of the equilenin molecule in the animal body. Doisy, *et al.* [1942] (58) have pointed out that dehydrogenation of some saturated ring structures has been effected by mammals but that the reduction of aromatic rings seems to occur less frequently. It was suggested therefore that these reduction products of estrogens may be anabolites rather than catabolites in estrogen metabolism.



Equilin



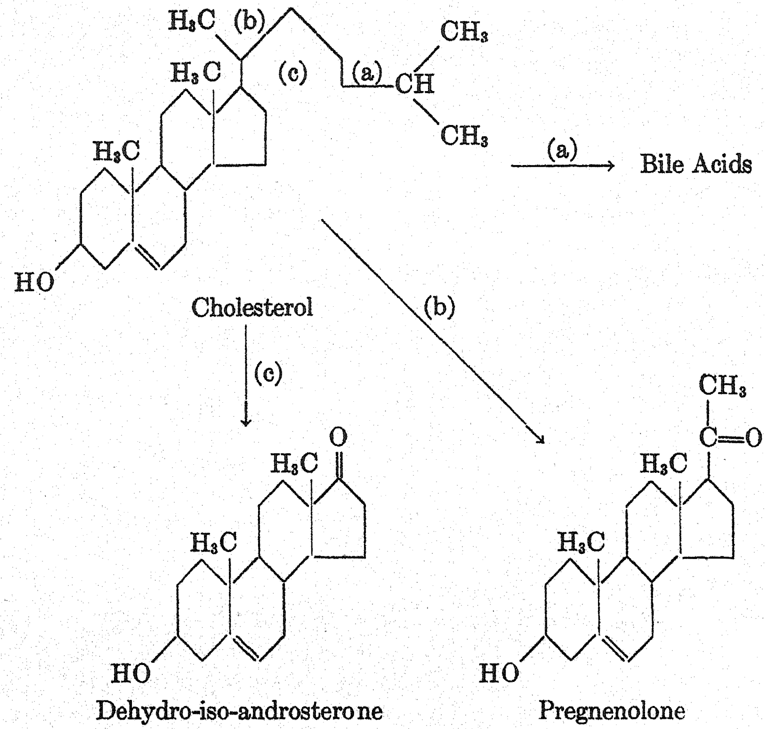
Equilenin



$\Delta^{5,7,9}$-Estratrienol-3-one-17

VII. ORIGIN AND INTERRELATIONSHIPS OF THE STEROIDAL HORMONES

Fieser [1937] (73) and Koch (119) have suggested that the formation of the sex hormones results by oxidative degradation of cholesterol *in vivo*. Attack at points (a), (b) and (c) of the steroid molecule (p. 336) would lead to the eventual formation of bile acids, progesterone and testosterone. Aromatization of ring A of steroids of the male sex hormone series might then give rise to phenolic estrogens.



There is ample experimental analogy for this type of oxidation; chromic acid oxidation of cholesterol will yield dehydroisoandrosterone and pregnenolone but the yields are very poor.

Marker [1938] (139) finds it difficult to understand how oxygen atoms, such as occur at C_{11} in cortical derivatives, or at C_{12} in bile acids, could be introduced into the cholesterol molecule. He has pointed out that attempts to show that cholesterol may be converted biochemically into the sex hormones have been inconclusive. Moreover, the drastic conditions which must be employed in the laboratory to oxidize the cholesterol side chain and the fact that phytosterols are not utilized in the animal organism throws grave doubt, in the opinion of this author, on the possibility of such oxidations in the animal organism. Koch [1939] (119) expressed the view that some of the sex hormones may be synthesized quite directly without passing through cholesterol. Marker [1938] (139) has designated $\Delta^{4,8}$-pregnadiendiol-17,21-trione-3,11,20 (or its hydrate at C_9) as a likely precursor of the sex hormones as well as of the hormones of the adrenal cortex. He believes moreover that this compound may yet be found in extracts of the adrenal cortex. His theory incorporates the hypothesis of side-chain oxidation of C_{21} steroids; aromatization of ring A is also postulated as a biological mechanism in the formation of estrogens. Koch [1939] (119) pointed out the highly speculative character of the assumption that estrogenic hormones originate from male hormones, since such processes call for specific removal of the methyl group in position 10. Although an increased excretion of estrogens after androgen administration has been observed, this may be due to a stimulation of estrogen production in the body. Some investigators have failed to observe an increased estrogen excretion under similar circumstances (213, 212, 120). On the other hand, the observation that the androgen, androstendione, also possesses estrogenic activity would seem to support the theory of ring aromatization, but Fieser (73) believes that this may be due to the inherent properties of the unsaturated diketone. Metabolic interrelationships within the C_{18} (estrogens), C_{19} (male sex hormones) and C_{21} (progesterone and its derivatives) steroid groups have been previously discussed in the text and require no further comment.

The fundamental question of the biosynthesis of the steroid skeleton, *i.e.*, of the perhydro-cyclopentenophenanthrene nucleus, still remains a most perplexing one. Rittenberg and Schoenheimer [1937] (187) suggested a synthesis of cholesterol from small molecules, possibly the intermediates of fat or carbohydrate metabolism; direct utilization of higher fatty acids to form the sterol molecule was considered quite improbable. Reichstein [1937] (185) suggested that these precursors might be triose molecules and indicated the manner in which cyclization might then occur. Sonderhoff and Thomas [1937] (211) demonstrated, with the aid of deuterium, that acetic acid is utilized by yeast in the synthesis of sterols, and Bloch and Rittenberg [1942] (12) found that the deuterium of the sodium acetate which had been fed to mice was incorporated into the cholesterol molecule. Since the cholesterol had a deuterium concentration far higher than would be expected if it had orignated in the body water, these investigators believe that acetic acid may act as a precursor in the biological formation of cholesterol. The isotope was shown to be present in both the side-chain and the nucleus of the cholesterol molecule (Bloch and Rittenberg, 1942) (12). The experimental data exclude propionic, butyric and succinic acids directly, and pyruvic and acetoacetic acids indirectly, as intermediates in the acetate-sterol conversion. Additional support was also obtained for the view that the higher fatty acids are not intermediates in sterol synthesis. Further investigation along these lines is highly desirable and may throw much light on the obscure point regarding the immediate precursors in the biosynthesis of the sex hormones.

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The Hormones of the Adrenal Cortex

By T. REICHSTEIN and C. W. SHOPPEE*

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This review deals with work on the chemistry of the steroids of the adrenal cortex and of closely related compounds which has appeared during the period late 1937–1942 (July). On account of the war, work from some countries can be included only up to late 1941. Of the earlier literature may be mentioned the two previous reviews compiled by one of us (1, 2), while accounts of the work performed during the last 4 years have been given in the articles by Kendall (3), Wintersteiner and Smith (4), Miescher (5), Mason (6), Callow (7), Freud, Laqueur, and Mühlbock (8), Verzàr (9), Koch (10), Kendall (11), Monnet (16), Tausk (12), Kamm and Pfiffner (13), Pfiffner (14), and Butenandt (15). Physiological activity can only be dealt with here in a very general manner; we therefore apologize to all those investigators whose work cannot be reported. The articles by Kendall (11), Kamm and Pfiffner (13), Pfiffner (14), and the thesis by Monnet (16), however, deal with physiological investigations in some detail.

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I. INTRODUCTION

1. Basic Biological Facts

Research on the hormones of the adrenal cortex is based on the following facts:

a. The adrenals are vital organs; in nearly all animals complete bilateral adrenalectomy leads to death in a few days.[1] Numerous post-operational insufficiency symptoms have been observed, but there is as yet no agreement as to which constitutes the primary cause of death.

b. The vital function is connected with the adrenal cortex, and appears to operate principally by delivery into the blood of a mixture of substances, since by injection of suitable cortical extracts adrenalectomized animals can be kept alive and the numerous insufficiency symptoms prevented or cured.

c. Investigation of active cortical extracts shows that the activity can be concentrated in those fractions which contain principally a mixture of relatively heavily oxygen-substituted steroids. A considerable number (twenty-eight in all: see Table I) of such steroids have been isolated as pure crystalline compounds; the structure and configuration of most of these are known in detail, while some have been prepared by partial synthesis. Some six or seven compounds have been found to be more or less active according to various methods of assay, in that they either prolong life in adrenalectomized animals or are able to prevent or cure single insufficiency symptoms.

2. Evaluation of Biological Activity

There is today no assay method which is generally recognized for the quantitative evaluation of substances possessing cortical activity; equally, no substance is accepted as a general standard of activity. These defects are partly an expression of the great difficulties encountered in the quantitative estimation of cortical activity. There is, indeed, no agreement as to whether the activity of any single compound equals that of good extracts; some authors believe this to be so, while others consider that a mixture of at least two and possibly more compounds is necessary. In the opinion of the reviewers, the major portion of the evidence available today supports the latter view. At the same time it becomes increasingly difficult to decide whether a substance is to be described as an active hormone of the adrenal cortex, because a substance may give a positive response in one method of assay and a negative response in another. The reviewers therefore prefer no longer to speak of "cortical activity," but rather to restrict themselves to the term "biological activity," always specifying the method of assay, since it depends largely on the arbitrary choice of the investigator as to which method of assay is regarded as "decisive."

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[1] Birds usually die within a few hours.

Some authors (*e.g.*, Hartman and Grollman) are of the opinion that the relative ability to prolong or maintain life in adrenalectomized animals should be recognized as the only decisive basis of assay. To this it may be objected that although the death of an experimental animal is a rather clearly recognizable phenomenon, it is certainly not always a simply conditioned phenomenon. Moreover, although the administration of certain substances can prolong life in adrenalectomized animals, such animals often react very differently from normal animals under given experimental conditions; they can be intensely deficient in certain respects without dying. Apart from these difficulties, other assay methods, which depend more on the quantitative determination of the degree of a single deficiency symptom, permit a deeper insight into the real physiological changes.

The most important deficiency symptoms which follow adrenalectomy, and which are susceptible of quantitative estimation, are:—

1. Disturbance of the Na^+, K^+, Cl^-, and water balance, (increased excretion of Na^+, Cl^-, and water; retention of K^+).
2. Increase of the urea content of the blood.
3. Asthenia (inefficiency of muscle).
4. Disturbance of carbohydrate metabolism (decrease in liver glycogen, smaller resistance toward insulin).
5. Reduction of resistance to various traumata (cold, mechanical or chemical shock).

Most methods of assay depend on the alleviation of such deficiency symptoms in adrenalectomized animals. Some, however, make use of normal animals.

3. *Methods of Assay*

Of assay methods previously described (1, 2), the most frequently employed are the test in adrenalectomized dogs, the survival test in rats, and the work tests in rats of Everse-de Fremery and of Ingle. By strict observation of the conditions specified in regard to diet (17), temperature, etc., these methods furnish reproducible results. In the survival test in rats, the proposal to use young animals, first made so far as we know by Hartman (18), has proved especially valuable because the increase in weight constitutes a good and additional criterion of activity (19, 20, 21). All these methods make use of adrenalectomized animals.

A considerable number of new assay methods has been developed, of which the following may be mentioned briefly. Sodium retention and lowering of the serum potassium level can be used not only in adrenalectomized animals but also in normal animals (22, 23, 24, 25), and healthy individuals. Assay methods depending on variations in carbohydrate metabolism have been described by Long *et al.* (26), Wells (27), Grattan and Jensen (28), and Thorn *et al.* (29). Special test methods based on the sensitivity of adrenalectomized animals toward shock induced by cold (30), drugs (31, 34) wounds (32), or other mechanical trauma (33), have been described by Swingle *et al.*, Selye *et al.*, and others.

These and other assay methods (cf. 11, 14) permit a partial but rather detailed qualitative differentiation between the various biologically active substances or fractions isolated from the adrenal cortex; this will be briefly referred to later.

II. SUMMARY OF STEROIDS ISOLATED IN A STATE OF PURITY FROM THE ADRENAL CORTEX

1. Methods of Isolation

In recent years whole glands rather than the dissected cortices have been used almost exclusively as starting material; this is also the case for the industrial preparation of the extracts used in clinical work. Nevertheless it is highly probable that the steroids originate from the cortex and not from the medulla.[2] The most important methods used for the preparation of concentrates have been described previously (1, 2, 3),[3] and no methods new in principle have been developed for the separation of the concentrates. The most effective method for the isolation of the individual components of the concentrates, namely chromatographic separation of the acetylated mixture, has been increasingly used (36, 37, 38, 39). In many cases difficulty is encountered in regenerating the free parent substances from the isolated acetyl derivatives, because the former are in general sensitive to both alkali and acid. All α-ketols are sensitive to alkali, while all substances with a hydroxyl group at C_{11} or C_{17} are sensitive to acids. For the hydrolysis of the acetates of sensitive substances, use of aqueous methanolic potassium hydrogen carbonate, preferably at 20°, has proved successful (40). If, apart from the hydroxyl of the α-ketol group, there is also present an acetylated hydroxyl at C_3 as in the acetates of (II), (III), and (IV), complete hydrolysis requires the use of potassium carbonate under analogous conditions (39), whereby some of the material is often destroyed.

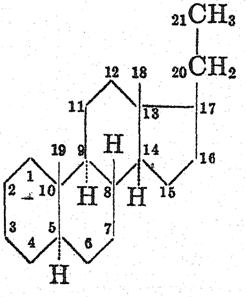
---

[2] Compare Bennett (35).

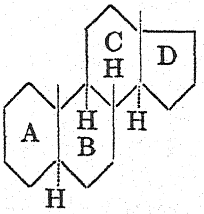
[3] Kendall (3) has published a more detailed description of his method of extraction.

2. *Nomenclature and Formulae*

Most of the steroids so far isolated from the adrenal cortex are derivatives of *allo*pregnane or Δ^4-pregnene; a few are derivatives of androstane. The stereochemical formulae and system of numbering of these two hydrocarbons are as follows:

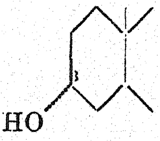


*allo*Pregnane

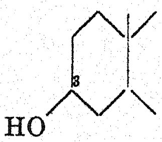


Androstane

When a carbonyl group is present as a substituent, it is sufficient to state its position (*e.g.* 3- or 20-); but for substituent hydroxyl groups (excluding a primary hydroxyl at C_{21}) two stereoisomerides are always possible. These will be distinguished (extending the proposal of L. F. Fieser (41)), by the indices α- and β-, the α-configuration being shown with a dotted valency-bond and regarded as below the plane of the ring concerned, the β-configuration being represented with a full valency-bond and regarded as above the plane of the ring.



3(α)-Hydroxy

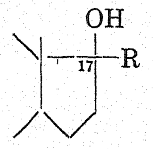


3(β)-Hydroxy

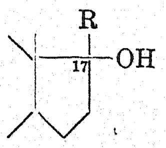
The indices α- and β- will also be used to distinguish stereoisomerism at C_{20}, where the isomerism is no longer geometrical in character (fixed position above or below the plane of a ring) but of the classical tartaric acid type (free rotation).

The assignment of configuration is in many cases not completely certain; moreover, even where good grounds exist for the assignment made, this is only relative to the configuration of another center of asymmetry. As yet the absolute configuration of no single center of asymmetry has been determined, so that if all the present assignments of configuration prove to be correct, it would still be possible for the stereochemical arrangement of the natural stereoids to be actually the mirror-image of that depicted above.

Of the various asymmetry centers, that at C_{17} is particularly important in connection with the steroids of the adrenal cortex. For 17-hydroxy compounds, membership of the 17(α)- or 17(β)-series has previously been expressed as follows:

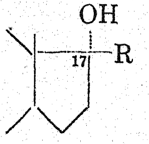


17(α)-Hydroxy

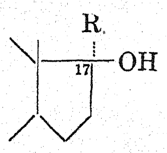


17(β)-Hydroxy

In order to make the spatial situation clearer, we shall employ the following representation in which, as before, a dotted valency-bond denotes position below the plane of the ring:



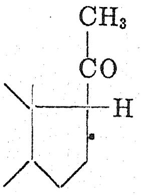
17(α)-Hvdroxy



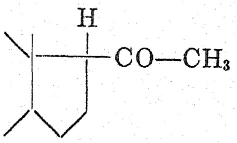
17(β)-Hydroxy

The spatial position of the side-chain is very uncertain in those pregnane-derivatives which possess no hydroxyl group at C_{17}. Several methyl ketones are known in both the stereoisomeric forms (42, 43, 44, 45), and are reversibly interconvertible by acids and alkalies, one form (the so-called "stable" form) predominating largely in the equilibrium mixture. Since, for example, the "stable" pregnenolone-acetate and the "stable" progesterone result by the direct oxidation of sterols with chromium trioxide,—admittedly in poor yield, but under conditions which tend to preclude inversion at C_{17},—it is to be assumed that in these two substances the $CH_3 \cdot CO$-group possesses the same configuration as that originally possessed by the side-chain of the sterols and bile-acids. According to Giacomello (46), X-ray measurements indicate that the long side-chain of the bile-acids is *cis* to the C_{18}-methyl group, although chemical evidence

(47) and optical rotatory values support the contrary view. On account of this general uncertainty, we shall represent the stable and labile ketones as follows, without any implication as to the real space distribution, and corresponding with the original formulation of Butenandt *et al.* (43). So far only the stable forms have proved to be biologically active.



stable form



labile form

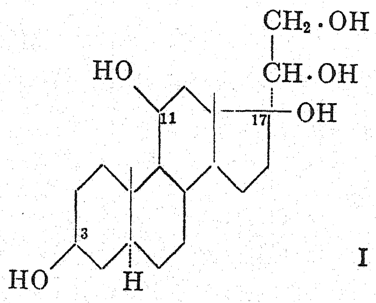
3. List of Steroids Isolated from the Adrenal Gland

(See Table I on page 352.)

4. Description of the Individual Compounds

a. $C_{21}O_5$ Group.

alloPregnane-3(β):11(β):17(β):20(?):21-pentol (**I**). $C_{21}H_{36}O_5$



I

First isolated by Wintersteiner and Pfiffner (48) and termed "Compound A," also obtained by Reichstein (49) and designated "Substance A," and by Kendall *et al.* (50) and called "Compound D." The substance crystallizes from ethanol or acetone in hexagonal plates, m.p. 221–222° (cor.); it takes up water of crystallization which is very difficult to remove. The specific rotation is $[\alpha]_D^{19} = +16° \pm 1°$ (ethanol) (49); $[\alpha]_{5461}^{25} = +29°$ (acetone) (50). The 3:20:21-triacetate is obtained by use of acetic anhydride and pyridine at 20°, m.p. 219–220°, $[\alpha]_D^{18} = +74° \pm 2°$ (acetone) (44). The constitution is established, also the configuration of most of the asymmetry centers. The β-configuration at C_3 is proved by direct conversion into androstane-3(β):17(α)-diol (52). The β-configuration at C_{11} is fixed by definition for the time being (39). Assignment

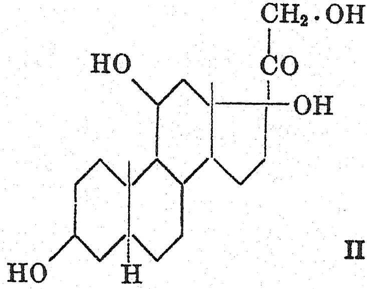
TABLE I

Steroids from the Adrenal Gland

No.	Empirical formulae	Systematic names*	Alphabetical and numerical designations		
			Reichstein *et al.*	Kendall *et al.*	Wintersteiner *et al.*
I	$C_{21}H_{36}O_5$	*allo*Pregnane-3(β):11(β):17(β):20(?):21-pentol	A	D	A
II	$C_{21}H_{34}O_5$	*allo*Pregnane-3(β):11(β):17(β):21-tetrol-20-one	V	—	—
III	$C_{21}H_{34}O_5$	*allo*Pregnane-3(α):11(?):17(β):21-tetrol-20-one	C	C	D
IV	$C_{21}H_{32}O_5$	*allo*Pregnane-3(β):17(β):21-triol-11:20-dione	D	G	? B
V	$C_{21}H_{32}O_5$	Δ^4-Pregnene-11(β):17(β):20(?):21-tetrol-3-one	E	—	—
VI	$C_{21}H_{30}O_5$	Δ^4-Pregnene-17(β):20(?):21-triol-3:11-dione	U	—	—
VII	$C_{21}H_{30}O_5$	Δ^4-Pregnene-11(β):17(β):21-triol-3:20-dione (17-Hydroxy-corticosterone)	M	F	—
VIII	$C_{21}H_{28}O_5$	Δ^4-Pregnene-17(β):21-diol-3:11:20-trione (17-Hydroxy-dehydrocorticosterone)	Fa	E	F
IX	$C_{21}H_{36}O_4$	*allo*Pregnane-3(β):17(β):20(β):21-tetrol	K	—	—
X	$C_{21}H_{34}O_4$	*allo*Pregnane-3(β):17(β):21-triol-20-one	P	—	—
XI	$C_{21}H_{30}O_4$	Δ^4-Pregnene-17(β):21-diol-3:20-dione	S	—	—
XII	$C_{21}H_{34}O_4$	*allo*Pregnane-3(β):11(?):21-triol-20-one	R	—	—
XIII	$C_{21}H_{32}O_4$	*allo*Pregnane-3(β):21-diol-11:20-dione	N	H	—
XIV	$C_{21}H_{30}O_4$	Δ^4-Pregnene-20(?):21-diol-3:11-dione	T	—	—
XV	$C_{21}H_{30}O_4$	Δ^4-Pregnene-11(?):21-diol-3:20-dione (Corticosterone)	H	B	—
XVI	$C_{21}H_{28}O_4$	Δ^4-Pregnene-21-ol-3:11:20-trione (11-Dehydrocorticosterone)	—	A	—
XVII	$C_{21}H_{28-30}O_4$	αβ-Unsaturated ketone: constitution unknown	—	—	—
XVIII	$C_{21}H_{36}O_3$	*allo*Pregnane-3(β):17(β):20(β)-triol	J	—	—
XIX	$C_{21}H_{36}O_3$	*allo*Pregnane-3(β):17(β):20(α)-triol	O	—	—
XX	$C_{21}H_{34}O_3$	*allo*Pregnane-3(β):17(β):diol-20-one	L	—	G
XXI	$C_{21}H_{30}O_3$	Δ^4-Pregnene-17(β)-ol-3:20-dione (17(β)-Hydroxy-progesterone)	—	—	—
XXII	$C_{21}H_{30}O_3$	Δ^4-Pregnene-21-ol-3:20-dione (11-Desoxycorticosterone)	Q	—	—
XXIII	$C_{21}H_{34}O_2$	*allo*Pregnane-3(β)-ol-20-one	—	—	—
XXIV	$C_{21}H_{30}O_2$	Δ^4-Pregnene-3:20-dione (Progesterone)	—	—	—
XXV	$C_{19}H_{30}O_3$	Androstane-3(β):11(β)-diol-17-one	—	—	—
XXVI	$C_{19}H_{24}O_3$	Δ^4-Androstene-3:11:17-trione (Adrenosterone)	—	—	—
XXVII	$C_{19}H_{26}O_2$	Δ^4-Androstene-3:17-dione	—	—	—

to the 17(β)-hydroxy-series is based on the use of optical superposition rules (39). The configurations of all other members of the $C_{21}O_5$ group are fixed by reference to this substance. In previous bioassays, the compound has proved to be inactive (48, 49, 50).

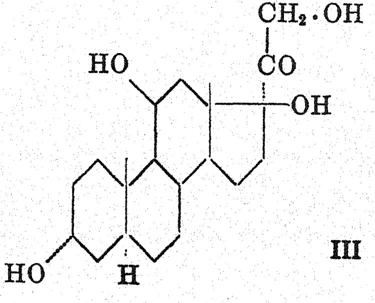
alloPregnane-3(β):11(β):17(β):21-tetrol-20-one (II). $C_{21}H_{34}O_5$



II

The isolation is described by von Euw and Reichstein (39) who term the compound "Substance V." It crystallizes from methanol-water in colorless needles containing water of crystallization, which become opaque at *ca.* 100°, m.p. 220–225° (cor., decomp.); it is not certain that this is the highest obtainable m.p. The specific rotation is $[\alpha]^{13}_{D} = +50.7° \pm 3°$, $[\alpha]^{13}_{5461} = +68° \pm 3°$ (dioxane). The substance gives a 3:21-diacetate, m.p. 225–227°, $[\alpha]^{18}_{D} = +62.6° \pm 2°$, $[\alpha]^{17}_{5461} = +77.3° \pm 2°$ (dioxane). Both the free hydroxyketone and its diacetate reduce alkaline silver solution rapidly and strongly at 20°; concd. sulphuric acid gives an immediate red-brown coloration. By cautious oxidation with chromium trioxide the diacetate is smoothly converted into that of (IV). The constitution and configuration are established, the latter by direct reference to the asymmetry centers of (I). The substance has not yet been examined for biological activity.

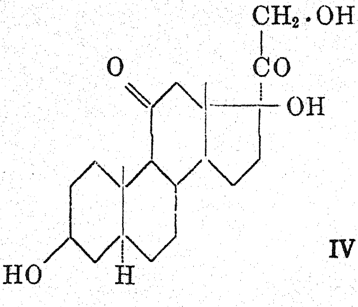
alloPregnane-3(α):11(?):17(β):21-tetrol-20-one (III). $C_{21}H_{34}O_5$



III

Isolated and described as "Compound D" by Wintersteiner and Pfiffner (48), isolated and named "Substance C" by Reichstein (49), isolated and called "Compound C" by Kendall *et al.* (50, 54), also isolated by Kuizenga and Cartland (53). The hydroxyketone separates from ethanol in fine needles; the purest specimens (39) have m.p. 273–276° (cor., decomp.), $[\alpha]^{15}_{D} = +73° \pm 4°$, $[\alpha]^{15}_{5461} = +90° \pm 4°$ (ethanol); $[\alpha]^{18}_{D} = +59° \pm 5°$, $[\alpha]^{18}_{5461} = +76° \pm 5°$ (dioxane). The 3:21-diacetate crystallizes from acetone-ether in rectangular leaflets, m.p. 204–205° (cor.), $[\alpha]^{15}_{D} = +73.8° \pm 2°$, $[\alpha]^{15}_{5461} = +90.5° \pm 2°$ (dioxane) (39). Both the free hydroxyketone and its diacetate reduce alkaline silver solution rapidly and strongly at 20°; concd. sulphuric acid furnishes an immediate red-brown color. The constitution is established, and the configuration nearly so, only the steric arrangement at C_{11} is undetermined. The substance possesses the α-configuration at C_3, and thus corresponds with androsterone; it is the only substance yet isolated from the adrenal gland which possesses this configuration at C_3. According to the dog method of assay and the Everse-de Fremery test the substance is inactive (48, 49, 50) in relatively small doses; Kuizenga and Cartland (53), however, report a considerable activity, *i.e.* 3–4 rat units per mg. in their rat method (20), an observation which clearly requires further investigation.

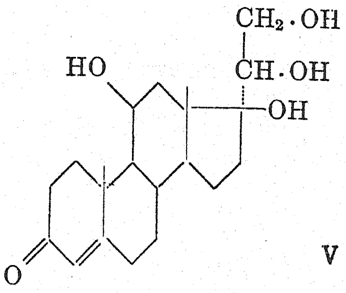
alloPregnane-3(β):17(β):21-triol-11:20-dione (IV). $C_{21}H_{32}O_5$



IV

Isolated and described as "Substance D" by Reichstein (49, 39), isolated and called "Compound G" by Kendall *et al.* (54), also isolated by Kuizenga and Cartland (53), and probably identical with "Compound B" of Wintersteiner and Pfiffner (48). The substance crystallizes from ethanol-acetone in glass-clear blocks, m.p. 238–242° (cor., decomp.) (39), $[\alpha]^{16}_{D} = +61.8° \pm 2°$, $[\alpha]^{16}_{5461} = +78.7° \pm 2°$ (dioxane) (39); $[\alpha]^{20}_{D} = +66° \pm 1.5°$ (ethanol) (49); $[\alpha]^{25}_{5461} = +83° \pm 2°$ (ethanol) (54). The 3:21-diacetate has m.p. 223–224° (cor.), $[\alpha]^{15}_{D} = +72.3° \pm 2°$, $[\alpha]^{15}_{5461} = +84.7° \pm 2°$ (dioxane) (39). Both the free hydroxyketone and the diacetate give a nearly colorless solution in concd. sulphuric acid, which soon becomes yellow. The substance reduces alkaline silver solution rapidly and strongly at 20°. The configuration is established by direct connection with (I) and (II). Bioassays show the substance to be inactive (48, 49, 53) in small quantities.

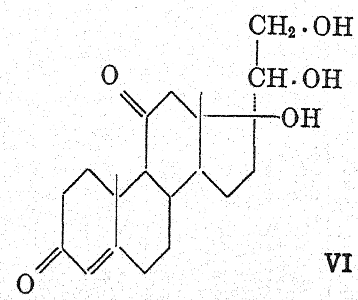
Δ⁴-Pregnene-11(β):17(β):20(?):21-tetrol-3-one (V). $C_{21}H_{32}O_5$



V

Isolated by Reichstein (49, 55, 38) and called "Substance E," the compound separates from acetone-water as a hydrate, m.p. 125° (unsharp). The monohydrate has $[\alpha]_D^{20} = +87° \pm 2°$ (ethanol) (49). Treatment with acetic anhydride and pyridine at 20° affords the 20:21-diacetate as colorless needles, m.p. 229–230°, $[\alpha]_D^{22} = +162.7° \pm 2°$ (acetone) (38). Careful oxidation of the diacetate leads to that of (VI) in good yield. The substance does not reduce alkaline silver solution at 20°, but gives with concd. sulphuric acid an orange solution which shows a green fluorescence when viewed on a dark background. The constitution and configuration are established (38, 39), the latter by direct transformation into (I) (38), from which it also follows that the undetermined configuration at C_{20} is the same as in (I). The substance has so far proved to be inactive.

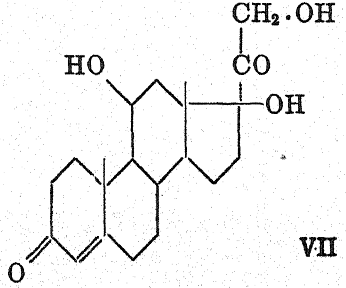
Δ⁴-Pregnene-17(β):20(?):21-triol-3:11-dione (VI). $C_{21}H_{30}O_5$



VI

This compound, isolated by Reichstein and von Euw (38), and called "Substance U," crystallizes from acetone-ether in stellate clusters of needles, m.p. 208° (cor.). The 20:21-diacetate has m.p. 252–253° (cor.), $[\alpha]_D^{21} = +178.5° \pm 2°$ (acetone). The constitution and configuration are proved (38, 39), while the stereochemical arrangement of the side-chain at C_{20} is the same as in (I). With concd. sulphuric acid, the substance furnishes an orange solution, which, viewed above a dark surface, shows a green fluorescence, which is much weaker than that given by (V) and its diacetate. Alkaline silver solution at 20° is either not reduced, or only very slowly, by the compound and its diacetate. The substance has not yet been examined for biological activity.

Δ^4-Pregnene-11(β):17(β):21-triol-3:20-dione; 17-Hydroxy-corticosterone (VII). $C_{21}H_{30}O_5$

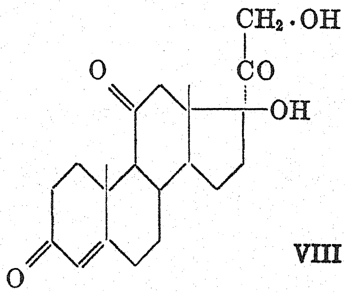


VII

Isolated and described as "Substance M" by Reichstein (55) and by Kendall *et al.* (54), who called it "Compound F," also isolated by Kuizenga and Cartland (53). The substance separates from ethanol in small prisms, striated cylinders, or cruciform aggregates, m.p. 207–210° (cor., decomp.), the m.p. observed depending on the rate of heating (55), $[\alpha]_D^{22} = +167.2° \pm 2°$ (ethanol). Kendall *et al.* report m.p. 217–220° (uncor.), $[\alpha]_{5461}^{25} = +178° \pm 2°$ (ethanol?). The 21-monoacetate (56) has m.p. 223–225° (cor.). Both the free hydroxyketone and its acetate reduce alkaline silver solution immediately at 20°. Concd. sulphuric acid furnishes an orange solution, which exhibits a very vivid green fluorescence when viewed above a dark surface. By careful oxidation, the monoacetate of (VII) can be transformed into that of (VIII) in good yield. The constitution and configuration are established (56, 54, 39). The substance is active in the Everse-de Fremery test, the unit being approximately 1.5–2 mg. (97). No precise figures are given for its activity in adrenalectomized dogs, but Mason (6) states that this is of the same order as that of (VIII), of which 7–10 mg. daily are necessary for a 15 to 20 kg. dog (54). Kuizenga and Cartland (53) found that it is definitely active in the survival test in rats but contains slightly less than 2 rat units (20) per mg. (subcutaneous injection). The compound is very active in Ingle's test (60,

98) and also in the test for anti-insulin (glycotropic) effect in mice (28). In normal dogs it increases the excretion of sodium and chloride (65).

Δ^4-Pregnene-17(β):21-diol-3:11:20-trione; 17-Hydroxy-dehydrocorticosterone (VIII). $C_{21}H_{28}O_5$



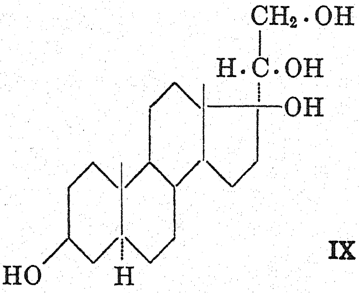
VIII

Isolated and described as "Compound F" by Wintersteiner and Pfiffner (57), isolated and called "Compound E" by Kendall *et al.* (50, 58), isolated and named "Substance Fa" by Reichstein (59), also isolated by Kuizenga and Cartland (53). The substance crystallizes from ethanol or acetone in colorless, glittering rhombohedra, m.p. *ca.* 215° (cor., decomp.) (dependent on crystal-size and rate of heating); $[\alpha]^{25}_{D} = +209° \pm 1°$ (95 per cent ethanol) (57); $[\alpha]^{25}_{5461} = +270° \pm 4°$ (benzene) (50, 58); $[\alpha]^{25}_{5461} = +248° \pm 4°$ (95 per cent ethanol) (58). The constitution and configuration are established (55, 56, 54, 39). The substance yields a 21-monoacetate, m.p. 239–241° (cor.) (56). The free hydroxyketone and its acetate both reduce alkaline silver solution immediately at 20°. Most specimens of the free compound and its acetate give with concd. sulphuric acid an orange solution showing a green fluorescence (57). The completely pure monoacetate (chromatographically purified) does not give this color reaction (39), which is probably caused by the presence of traces of (VII). The compound is little active in the test with dogs, the daily requirement for a 15 to 20 kg. dog being 7 to 10 mg. (54), and but little active in the Everse-de Fremery test (the unit is about 2 mg.). It also possesses only slight activity in the survival test in rats, the Cartland and Kuizenga unit (20) corresponding to approximately 1 mg. On the other hand it possesses high activity in those methods of assay which are related to carbohydrate metabolism. Long, Katzin and Fry (26), Wells (27), Wells and Chapman (62), Wells and Kendall (63) and others demonstrated its diabetogenic activity (increased glyconeogenesis) in rats and Grattan and Jensen (28) its anti-insulin effect in mice. Big doses produce glycosuria even in normal rats (64). As shown by Thorn *et al.* (65), in normal dogs it *increases* the excretion of sodium and chloride. It is very active in Ingle's test (60, 98), the activity being destroyed by reduction of the Δ^4-double bond (60).

b. $C_{21}O_4$ *Group.*

Sub-group *a*: Without an Oxygen Atom at C_{11}.

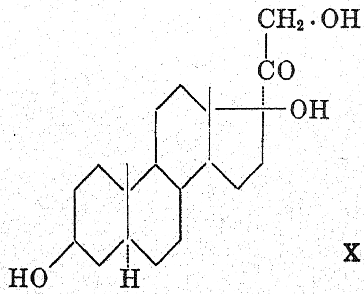
alloPregnane-3(β):17(β):20(β):21-tetrol[4] (IX). $C_{21}H_{36}O_4$



IX

Isolated by Steiger and Reichstein (36) and described as "Substance K," this compound crystallizes from methanol-water in colorless leaflets containing water of crystallization lost at *ca.* 140° when the crystals become opaque, m.p. 198–200° (cor.), $[\alpha]_D^{21} = -1° \pm 2°$ (ethanol). The 3:20:21-triacetate has m.p. 178–179° (cor.), $[\alpha]_D^{19} = +53.2° \pm 1°$ (acetone). The constitution and configuration are established (67, 68) and the partial synthesis from cholesterol has been accomplished (69, 70). The substance is inactive in all bioassay methods used hitherto.

alloPregnane-3(β):17(β):21-triol-20-one (X). $C_{21}H_{34}O_4$



X

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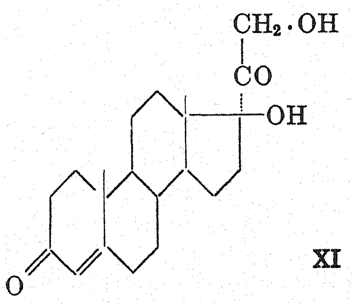
[4] For nomenclature relating to stereoisomerism at C_{20} see Prins and Reichstein (66).

Isolated by Steiger and Reichstein (36) and by Reichstein and Gätzi (67), "Substance P" separates from ethanol in colorless needles, m.p. 230–239° (cor., decomp.), $[\alpha]_D^{20} = +48° \pm 3°$ (ethanol). The 3:21-diacetate (36, 67) crystallizes from chloroform in needles, m.p. 208–209° (cor.) (39), $[\alpha]_D^{17} = +38.4° \pm 3°$ (66), $[\alpha]_D^{12} = +46.1° \pm 2°$ (71), $[\alpha]_{5461}^{17} = +53.8° \pm 3°$ (66) (chloroform); $[\alpha]_D^{16} = +28.2° \pm 4°$ (acetone) (72); $[\alpha]_D^{18} = +44.5° \pm 3°$, $[\alpha]_{5461}^{18} = +47.4° \pm 3°$ (dioxane) (39). The substance reduces alkaline silver solution rapidly at 20°; catalytic hydrogenation gives (IX) together with the isomeric *allo*pregnane-3(β):17(β):20(α):21-tetrol (67). The constitution and configuration are proved, and the partial synthesis from cholesterol has been carried out (71).

Δ^4-*Pregnene-17(β):21-diol-3:20-dione* (**XI**). $C_{21}H_{30}O_4$

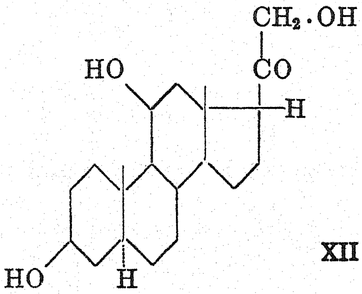


XI

Isolated by Reichstein and von Euw (37) and described as "Substance S" (73), the compound separates from ethanol in flat spearhead-shaped crystals, m.p. *ca.* 213° (cor., decomp.) dependent on the rate of heating; mixed m.p. determinations with the hydroxyketone (**VIII**) show no depression. The 21-monoacetate crystallizes from acetone in flattened needles, m.p. 239–241° (cor.), $[\alpha]_D^{19} = +116° \pm 4°$ (acetone), and gives no m.p. depression by admixture with the 21-monoacetate of (**VIII**), m.p. 239–241°. Apart from analysis, the color reaction with concd. sulphuric acid, whereby (**XI**) and its monoacetate furnish a pure red coloration, serves for differentiation. Constitution and configuration are proved and the partial synthesis from cholesterol has been accomplished (74, 75). The monoacetate of (**XI**) is active in the Everse-de Fremery test, in which *ca.* 1 mg. corresponds to a unit, and also in the survival test in rats as judged by the survival of the animals in the Everse-de Fremery test. It proves, however, to possess very little or no activity in Ingle's test (61) and in the anti-insulin test of Grattan and Jensen (76).

Sub-group *b*: With an Oxygen Atom at C_{11} (Corticosterone Group).

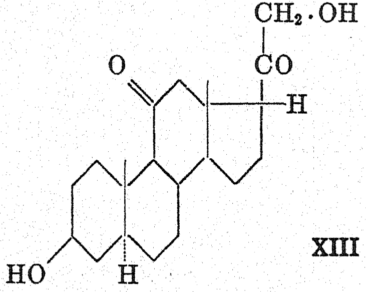
alloPregnane-3(β):11(?):21-triol-20-one (**XII**). $C_{21}H_{34}O_4$



XII

Isolated by Reichstein and von Euw (37, 73) and called "Substance R," the compound crystallizes from ethanol in colorless needles, m.p. 202–204° (cor.). It gives a 3:21-diacetate, m.p. 173–174°, $[\alpha]_D^{17} = +83.7° \pm 2°$, $[\alpha]_{5461}^{17} = +102.7° \pm 2°$ (dioxane); $[\alpha]_D^{18} = +92.4° \pm 2°$, $[\alpha]_{5461}^{18} = +114.3° \pm 2°$ (acetone) (39). The substance reduces alkaline silver solution strongly and immediately at 20°. The constitution is established, also the configuration at C_3; on the other hand, the configuration at C_{11} is not yet determined. By oxidation as the diacetate with chromium trioxide, (**XII**) yields the diacetate of (**XIII**). The substance has not been submitted to bioassay.

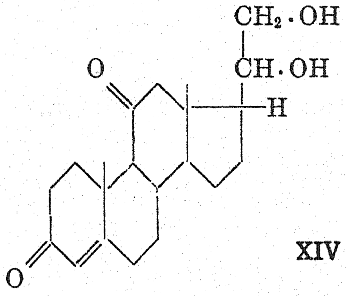
alloPregnane-3(β):21-diol-11:20-dione (**XIII**). $C_{21}H_{32}O_4$



XIII

Isolated and described as "Compound H" by Kendall *et al.* (77), and by Steiger and Reichstein (36) who named it "Substance N." According to Kendall *et al.* the compound has m.p. 172–176° (uncor.), $[\alpha]_{5416}^{25} = +118°$ (ethanol), whereas Steiger and Reichstein give m.p. 189–191° (cor.), $[\alpha]_D^{19} = +93.8° \pm 2°$ (ethanol). The 3:21-diacetate (67, 73) has m.p. 144–145° (cor.), $[\alpha]_D^{18} = +77.5° \pm 2.5°$, $[\alpha]_{5461}^{18} = +99.5° \pm 2.5°$ (acetone) (44); $[\alpha]_D^{19} = +85.6° \pm 2°$, $[\alpha]_{5461}^{19} = +105.6° \pm 2°$ (dioxane) (39). The substance can be prepared by a simple series of reactions from (**I**), which proves the configuration at C_3 (44); the constitution had been established previously (77). No results in biological tests have been reported.

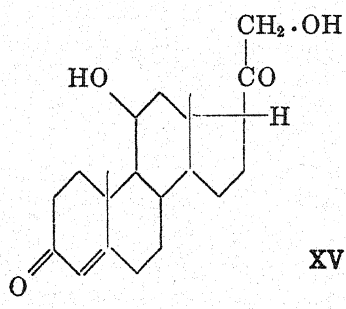
Δ⁴-Pregnene-20(?):21-diol-3:11-dione (**XIV**). $C_{21}H_{30}O_4$



XIV

Isolated and described as "Substance T" by Reichstein and von Euw (78), the free hydroxyketone, crude m.p. *ca.* 210° (cor.), was not purified. The 20:21-diacetate has m.p. 212–213°. The constitution is established, but the configuration at C_{20} undetermined. The substance has not been submitted to bioassay.

Δ⁴-Pregnene-11(?):21-diol-3:20-dione; Corticosterone (**XV**). $C_{21}H_{30}O_4$



XV

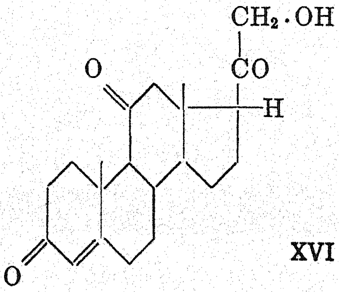
Isolated by Reichstein (55), the compound was first designated as "Substance H" and later named "corticosterone"; the substance was at the same time isolated by Kendall *et al.* (77), who first termed it "Compound B" and later called it "corticosterone." It was also isolated by Kuizenga and Cartland (79).

The substance crystallizes from acetone in plates, m.p. 180–182°, $[\alpha]_D^{15} = +223° \pm 3°$ (ethanol) (55); $[\alpha]_D^{25} = +222°$, $[\alpha]_{5461}^{25} = +258° \pm 3°$ (ethanol) (77). The 21-monoacetate has m.p. 145–146° (cor.), generally followed by crystallization and remelting at 153° (cor.); $[\alpha]_D^{18} = +200° \pm 2°$, $[\alpha]_{5461}^{18} = +245° \pm 2°$ (dioxane) (39). A series of other esters have been described by Reichstein (55), and by Kuizenga and Cartland (79).

The substance and its esters reduce alkaline silver solution strongly and immediately at 20°; with concd. sulphuric acid, they yield orange solutions which show an intense green fluorescence when viewed above a dark surface. The substance is markedly decomposed by OH^- at 20°, and warm mineral acids also cause decomposition. By cautious oxidation as the monoacetate, (**XV**) can be converted into (**XVI**). The constitution is established, but the configuration at C_{11} is undetermined. The substance is active in the Everse-de Fremery test, approximately 1 mg. corresponding to the unit (80); it is also active in Verzár's glucose test in rats with 0.2 mg. (81), and in Ingle's test with 0.5 mg. (60). In the dog test (80), a daily dose of 1.5–2 mg. sufficed to maintain an adrenalectomized 13.6 kg. dog in good condition, although 1 mg. daily was not sufficient (3, 6). In their survival test in rats (20), Kuizenga and Cartland (79) report an activity of 6 units per mg. The effective dose is the same by subcutaneous injection and administration by stomach tube (82). Several esters were found to be more active than free corticosterone (79), the diethylacetate, m.p. 179°, being four times, and the butyrate, m.p. 168°, three times as active as free corticosterone (injection technique); esters of higher fatty acids were less active. Long *et al.* (26), Wells (27), Thorn *et al.* (83, 84) and others have demonstrated the effect of corticosterone on carbohydrate metabolism in animals and human subjects. Corticosterone and its acetate also exert a pronounced anti-insulin effect (28) in mice. The sodium and chloride retaining effect of corticosterone is established (25, 65) but it seems to have little effect in lowering the serum potassium (22, 63). Selye and Dosne (32) have demonstrated its effect in combating shock induced by surgical trauma and other means. Desoxycorticosterone (**XXII**) was ineffective under similar conditions.

Hydrogenation of the Δ^4-double bond largely destroys the biological activity as tested by Ingle's method (77). Of all the pure compounds examined the nearest qualitative approach to the full activity of adrenal cortical extracts is shown by (**XV**), (**XVI**), and their esters.

Δ^4-*Pregnene-21-ol-3:11:20-trione*; *Dehydrocorticosterone* (**XVI**). $C_{21}H_{28}O_4$



XVI

Isolated and described as "Compound A" by Kendall *et al.* (50, 77), isolated and described as (11)-dehydrocorticosterone by Reichstein and von Euw (37), also isolated by Kuizenga and Cartland (53). The substance crystallizes from aqueous acetone in prisms, m.p. 177–180° (uncor.) (50, 77); 178–180° (cor.) (53), $[\alpha]_{5461}^{25} = +299° \pm 1°$ (ethanol) (37); $[\alpha]_{5461}^{25} = +347°$ (benzene) (50). The 21-monoacetate (55) has m.p. 179–181°, $[\alpha]_{D}^{18} = +233.7° \pm 2°$, $[\alpha]_{5461}^{18} = +285.1° \pm 2°$ (dioxane) (39). The substance reduces alkaline silver solution immediately at 20°, but gives no green fluorescence with concd. sulphuric acid; it is stable to hot dilute mineral acids, but rather rapidly decomposed by OH^-.

11-Dehydrocorticosterone is active in the survival test in rats; Kuizenga *et al.* (53, 82) found approximately 4 rat units per mg. by subcutaneous injection and about 6 R.U. per mg. when administered by stomach tube (82). It is also active in Ingle's test (60), and Long *et al.* (26) demonstrated its diabetogenic effect. It produces sodium and chloride retention (25, 65). The degree of biological activity shown in most assay methods closely resembles that exhibited by (XV); similarly, the activity in the dog test is destroyed almost completely by hydrogenation of the Δ^4-double bond (77).

Sub-group *c.*

αβ-Unsaturated Ketone $C_{21}H_{28-30}O_4$ (XVII) *of Unknown Constitution*

Pfiffner and North (85) have isolated an *αβ*-unsaturated ketone, m.p. 261–264° (uncor.), $[\alpha]_{D}^{23} = +133° \pm 4°$ (chloroform); it does not reduce alkaline silver solution, and gives no color with concd. sulphuric acid. It is stable to acids and to OH^- in small concentration, and yields a monoacetate, m.p. 208–210°. Oxidation with chromium trioxide furnishes a neutral compound, $C_{21}H_{26\ 28}O_4$, m.p. 206–208°, which still contains a reactive carbonyl group. It is difficult to reconcile these properties with a formula analogous in type to those of the other substances isolated from the adrenal cortex. The ketone (0.25 mg.) is inactive in the survival test in rats. In doses of 2 mg. it exhibits no progestational activity and in doses of 1.5 mg. exerts no androgen activity in castrated rats.

c. $C_{21}O_3$ *Group.*

alloPregnane-3(β):17(β):20(β)-triol (XVIII).[5] $C_{21}H_{36}O_3$

Isolated by Reichstein (59) and described as "Substance J," this compound separates from moist acetone in needles as a hydrate; the crystals lose water, becoming opaque at 120–130°, and have m.p. 216–217° (cor.),

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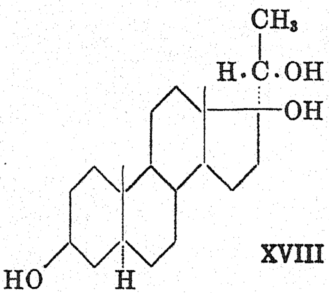
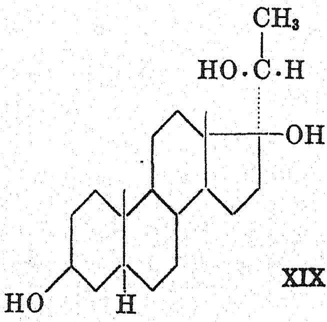
[5] In connection with the nomenclature at C_{20} see Prins and Reichstein (66).



XVIII

$[\alpha]_D^{19} = -7.9° \pm 1°$ (ethanol) (36). The 3:20-diacetate has m.p. 159–160° (cor.), $[\alpha]_D^{19} = +24.6° \pm 1°$ (acetone) (36). The constitution and configuration are established, a partial synthesis has been carried out (86, 87).

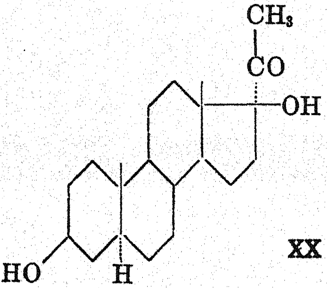
alloPregnane-3(β):17(β):20(α)-triol (XIX). $C_{21}H_{36}O_3$



XIX

Isolated by Steiger and Reichstein (36) and described as "Substance O," this compound crystallizes from methanol-water in leaflets as a hydrate; the crystals lose water and become opaque at *ca.* 130°, m.p. 222–223° (cor.), $[\alpha]_D^{20} = -12.6° \pm 2°$ (methanol). The 3:20-diacetate has m.p. 250° (cor.), $[\alpha]_D^{21} = -30.1° \pm 2°$ (acetone) (36). The constitution and configuration are established; thus (**XVIII**) and (**XIX**) differ solely in the stereo-chemical arrangement at C_{20}. A partial synthesis has been accomplished (87). The substance has not been tested for biological activity.

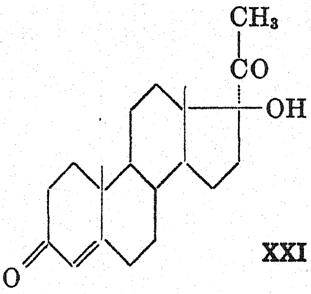
alloPregnane-3(β):17(β)-diol-20-one (XX). $C_{21}H_{34}O_3$



XX

Isolated and described as "Compound G" by Wintersteiner and Pfiffner (57), also isolated and designated as "Substance L" by Reichstein (59). The substance is very difficultly soluble in most organic solvents, and has m.p. 264° (cor., decomp.), $[\alpha]_D^{26} = +38° \pm 1°$ (57), $[\alpha]_D^{21} = +30.6° \pm 3°$ (88) (ethanol). The constitution and configuration are established; partial syntheses have been accomplished from (X) (89), and in other ways (90, 91).

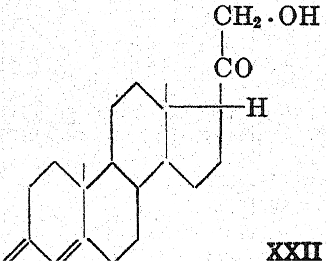
Δ^4-*Pregnene-17(β)-ol-3:20-dione; 17(β)-Hydroxy-progesterone* (**XXI**). $C_{21}H_{30}O_3$



XXI

The compound was first isolated by Pfiffner and North (92, 93), who reported m.p. 212–215° (uncor.), $[\alpha]_D^{27} = +102° \pm 3°$ (chloroform). Subsequently, the substance was also isolated by von Euw and Reichstein (94), who found m.p. 222–223° (cor.) with not too slow heating; with slow heating, the substance undergoes molecular rearrangement accompanied by partial resolidification, and becomes completely molten only at 276°; $[\alpha]_D^{17} = +105.6° \pm 2°$ (chloroform). The constitution and configuration are established, and the partial synthesis is accomplished (91, 90, 15). The substance is inactive in doses of 0.25 mg. in the survival test in young rats, also in Ingle's test with doses up to 3 mg., and has no progestational activity. On the other hand it is androgenic in castrated rats, the activity being of the same order as that of androsterone and adrenosterone (**XXVI**). In the comb-growth test a daily dose of 0.2 mg. is ineffective (injection technique?) (92, 93), in contrast to the positive result obtained by direct application to the comb (15).

Δ^4-*Pregnene-21-ol-3:20-dione; Desoxycorticosterone* (**XXII**). $C_{21}H_{30}O_3$



XXII

Isolated by Reichstein and von Euw (37) and designated "Substance Q" or desoxycorticosterone, the substance had previously been obtained by partial synthesis (95); it crystallizes from ether in colorless plates, m.p. 141–142° (cor.), $[\alpha]_D^{22} = +178° \pm 3°$ (ethanol). The 21-acetate (Doca[6]) has m.p. 157–159°, $[\alpha]_D^{19} = +177° \pm 4°$ (ethanol); $[\alpha]_D^{18} = +164.3° \pm 2°$, $[\alpha]_{5461}^{18} = +199.8° \pm 2°$ (acetone) (39); $[\alpha]_D^{19} = +173.6° \pm 2°$, $[\alpha]_{5461}^{19} = +211.9° \pm 2°$ (dioxane) (39). A large number of other esters have been described (96). The substance and its esters reduce alkaline silver solution immediately at 20°; both it and they are sensitive to OH^-, but relatively stable to acids. The constitution is established and various partial syntheses have been carried out (*cf.* pp. 395–397). Because the substance is a commercial product, and today is much used clinically in the form of its acetate, the latter has been the subject of many physiological investigations; only a few results can be mentioned here.[7] In the dog method of assay (6), in the Everse-de Fremery test (97), in the survival test in rats (82), and in sodium and chloride retention (25, 65), desoxycorticosterone and its esters are the most effective of all crystalline compounds so far known. On the other hand, they have little or no activity in Ingle's test (98, 61), and in those methods of assay which are based on carbohydrate metabolism (26, 28). The results obtained in inhibiting surgical or chemical shock are erratic. A few figures may be picked out from the great number of investigations: Mason (6) states that for a 20 kg. dog the daily requirement of desoxycorticosterone is about 0.3 mg. and of the acetate about 0.1 mg. when given in a single dose (corticosterone 2 mg., and compound (**VIII**) about 7 to 10 mg. under the same conditions). In the Everse-de Fremery test a unit corresponds to approximately 0.07 mg. of the acetate (97); the free hydroxyketone is several times less active. Grollman found the necessary daily dose in his survival test in rats (99) to be about 1 mg., both by subcutaneous injection and by oral administration in the drinking water (100), while Kuizenga *et al.* (82) found in their rat method of assay (20) about 35 Rat Units per mg. when injected and less than 1 R.U. when given by stomach tube (see footnote 18). Sodium, chloride and water retention can go so far as to produce edema and other toxic symptoms (101). The acetate has slight but distinct progestational activity equivalent to about $\frac{1}{6}$ to $\frac{1}{10}$ that of progesterone (96, 102, 103, 104). It has been shown that in the human organism (104) and in rabbits (105) the substance is converted into pregnane-3(α):20-diol, which is excreted in the urine as the glucuronide; reduction of the hydroxyl group at C_{21} thus occurs, but whether or not this precedes reduction of ring A is uncertain. It is therefore possible that the slight progestational activity possessed by desoxycorticosterone is to be traced to its conversion, to a limited extent, into progesterone (**XXIV**).

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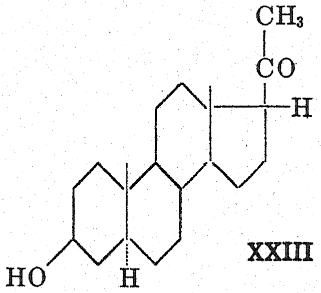
[6] This is a convenient synonym; it may be remarked that there is often failure, especially in physiological papers, to distinguish between the acetate and the free hydroxyketone.

[7] Besides the reviews mentioned above, a good summary dealing especially with DOCA is given by G. A. O. in "Het Hormoon" **9,** (No. 10) 201 (1941).

d. $C_{21}O_2$ Group.

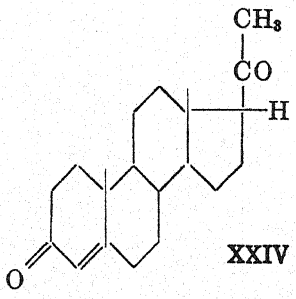
alloPregnane-3(β)-ol-20-one (**XXIII**). $C_{21}H_{34}O_2$



XXIII

Originally obtained from the *Corpus luteum*, this compound has been isolated from the adrenal cortex by Beall and Reichstein (106, 107). Its properties are known, the constitution and configuration are established, and the partial synthesis has been carried out. No biological activity has been observed.

Δ⁴-Pregnene-3:20-dione; Progesterone (**XXIV**). $C_{21}H_{30}O_2$



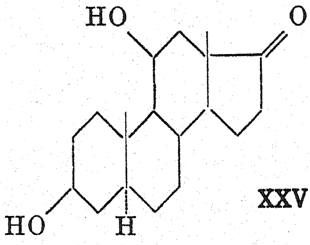
XXIV

This substance, previously isolated from the *corpus luteum* and characteristic for the physiological activity of that gland, has also been isolated by Beall and Reichstein (106, 107) from the adrenal gland. The constitution is established and the partial synthesis accomplished. Apart from its known progestational activity, the compound is able to maintain adrenalectomized rats and ferrets in daily doses of 1–4 mg. (108, 109, 110).

It is inactive in Ingle's test (111) and in the anti-insulin test (28) but has some small activity in sodium and chloride retention (112).

e. Steroids with Less Than 21 Carbon Atoms

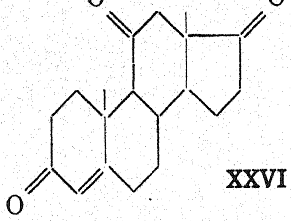
Androstane-3(β):11(β)-diol-17-one (**XXV**). $C_{19}H_{30}O_3$



XXV

The compound was first obtained by degradation of (I) (113), but later isolated in small quantity directly from extracts of the adrenal cortex (37, 94). It is uncertain whether it occurs in the completely fresh gland, and it is possible that it originates during working up by oxidation or decomposition of substances with 21 carbon atoms. The compound separates from acetone-ether in colorless needles, m.p. 234–235° (cor.), $[\alpha]_D^{20} = +84.5° \pm 3°$ (ethanol); $[\alpha]_D^{19} = +81.3° \pm 2°$, $[\alpha]_{5461}^{19} = +105° \pm 2°$ (dioxane) (39). The 3-mono-acetate (114, 94) crystallizes from acetone-ether in needles, m.p. 228–229° (cor.) (39), $[\alpha]_D^{19} = +70.5° \pm 2°$, $[\alpha]_{5461}^{19} = +87.1° \pm 2°$ (dioxane) (39). The free hydroxyketone is androgenic (113).

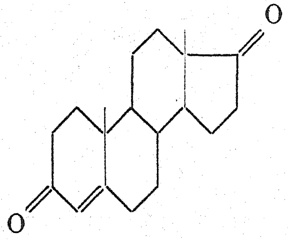
Δ^4-*Androstene-3:11:17-trione; Adrenosterone* (**XXVI**). $C_{19}H_{24}O_3$



XXVI

The compound was isolated by Reichstein (49, 115), and is obtained by degradation of the Δ^4-unsaturated members of the $C_{21}O_5$-group (59, 58, 55). Whether or not the compound occurs in the completely fresh gland is uncertain, since here also formation during working up is possible. The triketone forms colorless leaflets from acetone-ether, m.p. 223–224° (cor.), $[\alpha]_D^{20} = +262° \pm 5°$, $[\alpha]_{5461}^{25} = +364° \pm 5°$ (ethanol) (58). It is androgenic (115).

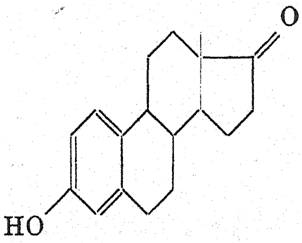
Δ^4-Androstene-3:17-dione (**XXVII**). $C_{19}H_{26}O_2$



XXVII

This previously known androgenic substance was isolated from the adrenal cortex in small amounts by von Euw and Reichstein (94); it is possible that it originates by oxidation of (**XI**) during working up.

Estrone (**XXVIII**). $C_{18}H_{24}O_2$



XXVIII

Already known for a considerable period, the compound was isolated by Beall (116) from the adrenal cortex and may be at least partly responsible for the estrogenic activity (122) of extracts of the adrenal cortex.

f. Other Substances

A number of nonsteroid compounds have been isolated from adrenal glands; these are probably unimportant in regard to biological activity[8] and will not be dealt with here. The following may be mentioned: leucylproline (57), $\beta\beta$-dihydroxydiethyl-sulphoxide (49, 117), dimethylsulphone (118), a base $C_{12}H_{12}O_2N_2$ (119) and an acid $C_{12}H_{20}O_3N_2$ (120).

The presence in adrenal extracts of substances possessing gonadotropic (121), lactogenic ("cortilactin") (122) and other physiological activity has been indicated by biological methods; since the substances responsible are possibly of protein character, the matter will not be considered here.

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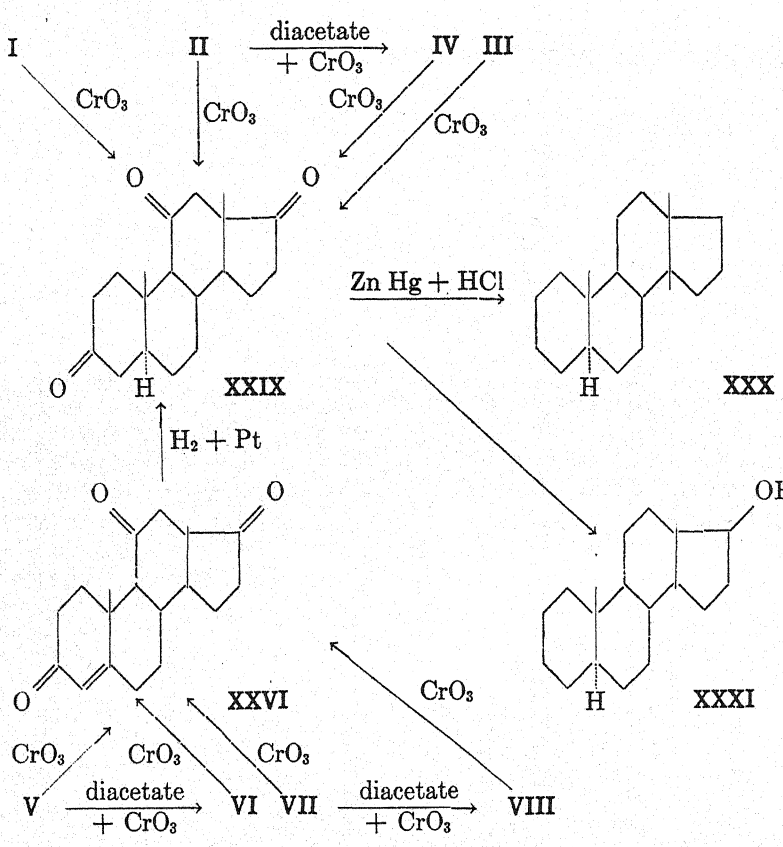
[8] It is not impossible that the fats and fatty acids present in natural extracts can act as activators. Thus, Miescher *et al.* (96) found that palmitic acid increased the activity of desoxycorticosterone in the survival test in rats.

III. Determination of Constitution and Configuration and Reciprocal Interconversions

1. *$C_{21}O_5$ Group*

The evidence for the constitution of some of the steroids isolated from the adrenal cortex has been given in previous reviews (1–14); in the meantime some uncertainties have been eliminated.


I
II
diacetate + CrO_3
IV
III
CrO_3
CrO_3
CrO_3
CrO_3
O
O
O
H
XXIX
Zn Hg + HCl
H
XXX
H_2 + Pt
O
O
OH
O
XXVI
CrO_3
H
XXXI
CrO_3
CrO_3
CrO_3
V
diacetate + CrO_3
VI
VII
diacetate + CrO_3
VIII

Special difficulties confronted the accurate location of the oxygen atom placed at C_{11}. A hydroxyl group at C_{11} is extremely unreactive, and can be acetylated only with great difficulty or not at all. As yet no carbonyl group at C_{11} has been characterized by the preparation of ketonic derivatives.[9] This extreme unreactivity can nevertheless only be used indirectly as a criterion of the presence of an oxygen atom at C_{11}, because, so far, no steroid derivatives proved to possess a hydroxyl group or a carbonyl group at C_{11} are available for comparison.[10]

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[9] The C_{11}-keto group survives extended treatment with sodium-potassium alloy in hot petroleum ether (114).

Summarizing the statements made above, the proof of the interrelation of all the substances of the $C_{21}O_5$ group and of their steroid character is as shown on p. 370.

Oxidation with chromium trioxide of (I), (II), (III) and (IV) yielded one and the same triketone (XXIX), which was converted by Clemmensen reduction (amalgamated zinc and hydrochloric acid) into the known hydrocarbon androstane (XXX) (136). At the same time some androstane-17(β)-ol (XXXI) was formed, whereby the presence of an oxygen atom at C_{17} in (XXIX) is proved. A strict proof of the presence of an oxygen atom at C_3 has been given by Shoppee (52) (p. 372).

Degradation of (I) with periodic acid afforded the dihydroxyketone (XXV), whose 3-monoacetate (XXXII) was dehydrated with hydrochloric acid to an anhydro-compound (XXXIII),[11] which by catalytic hydrogenation and subsequent acetylation furnished the known androstane-3(β):17(α)-diol-diacetate (XXXIV). This degradation not only proves the position of the oxygen atom at C_3, but simultaneously the β-configuration of the hydroxyl group at C_3 in (I). An analogous degradation has been performed on corticosterone (XV) in the $C_{21}O_4$ series whereby *allo*pregnane-3:20-dione is obtained; since a direct interrelation of the $C_{21}O_5$ group with the $C_{21}O_4$ group (Sub-group *b*) has been accomplished (p. 377), this constitutes a further proof of the presence of an oxygen atom at C_3.

Following the proof that the triketone (XXIX) possesses two carbonyl groups at C_3 and C_{17}, respectively, the previous experiments and considerations supporting the conclusion that the third carbonyl group must be at C_{11} became of increased validity. All positions other than C_{11} can be excluded on the following grounds. It is certain that the unreactive oxygen atom in (XXIX) is present as a keto-group because this is formed by oxidation of a hydroxyl-group with chromium trioxide (114), (compare earlier experiments by Kendall *et al.* (77) on several degradation products of the $C_{21}O_4$ group, Sub-group *b*); for a keto-group only positions 1, 2, 4, 6, 7, 11, 12, 15 and 16 have to be considered. Positions 1, 2, 4, 15 and 16 can be excluded at once, since otherwise (XXIX) must be either an α-diketone

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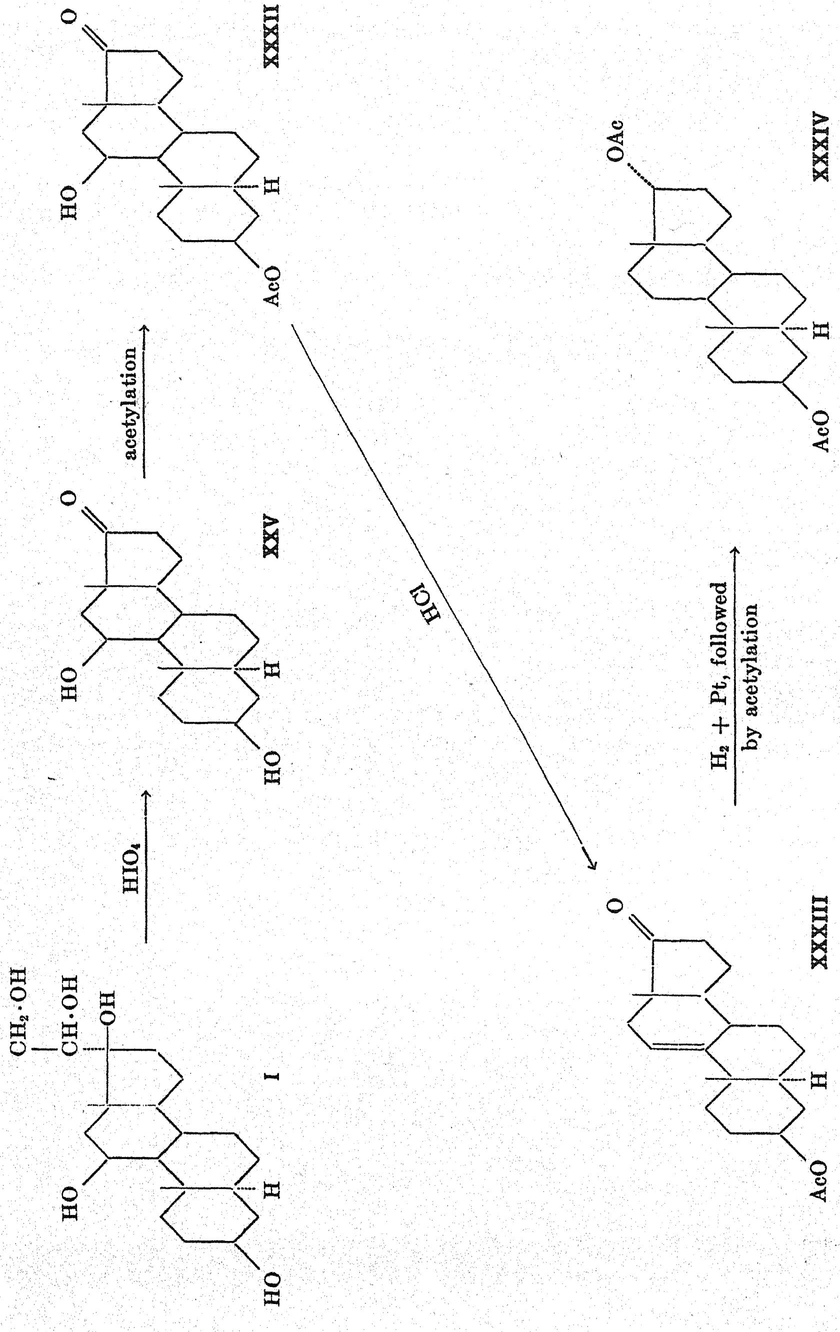
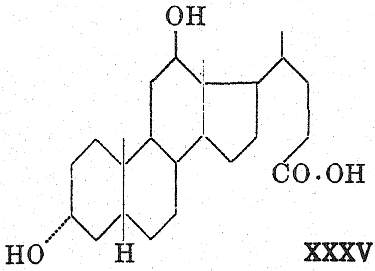
[10] 11-Hydroxy-12-ketocholanic acid and 11:12-diketocholanic acid are known (123, 124, 125, 126, 127, 128, 129), but here the adjacent carbonyl group at C_{12} appears to exercise a far-reaching influence on reactivity at C_{11}. Hydroxyl groups at C_{11} were assumed to be present in digoxigenin and sarmentogenin (130, 131); in the case of digoxigenin, this has been proved to be incorrect (132, 133, 134, 135) and while sarmentogenin may be a true C_{11}-hydroxy compound, this is not proved.

[11] Formulated in the original paper as Δ^{11}-unsaturated; the $\Delta^{9:11}$-formula (XXXIII) given here is not proved, but appears more probable in the light of recent unpublished work.

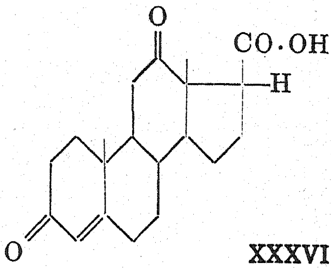

CH₂·OH
CH·OH
OH
HO
HO
H
I
HIO₄
HO
HO
H
O
XXV
acetylation
HO
AcO
H
O
XXXII
HCl
O
AcO
H
XXXIII
H₂ + Pt, followed by acetylation
OAc
AcO
H
XXXIV

or an alkali-soluble β-diketone, which is not the case. There remain only positions 6, 7, 11 and 12.

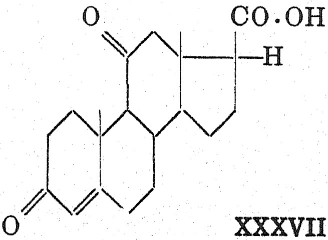
In the case of position 12, a non-enolizable β-diketone must result, and there is experimental evidence (114) against this; moreover, position 12 is excluded definitely by the work of Mason and Hoehn (133), who prepared the acid (**XXXVI**) by degradation of desoxycholic acid (**XXXV**), and showed it to be different[12] from the corresponding acid (**XXXVII**) from corticosterone. The positions 6 and 7 were improbable from the first, because oxygen atoms in these positions possess no special unreactivity. Further, 6- and 7-hydroxy and 6- and 7-carbonyl compounds are now known (137, 138, 139), which can be compared directly with isomerides from the adrenal cortex, and which are different from the latter. Positions 6 and 7 are therefore excluded, and only position 11 remains. All the results are consistent with the adoption as correct of position 11 for the unreactive oxygen atom.



XXXV



XXXVI



XXXVII

The proof of the position of the side chain at C_{17} follows from the conversion described on page 378 of (I) into (**XIII**), for which the *allo*-pregnane skeleton is established.

The four Δ^4-unsaturated members of the $C_{21}O_5$ group all yielded by oxidation one and the same Δ^4-unsaturated triketone (adrenosterone)

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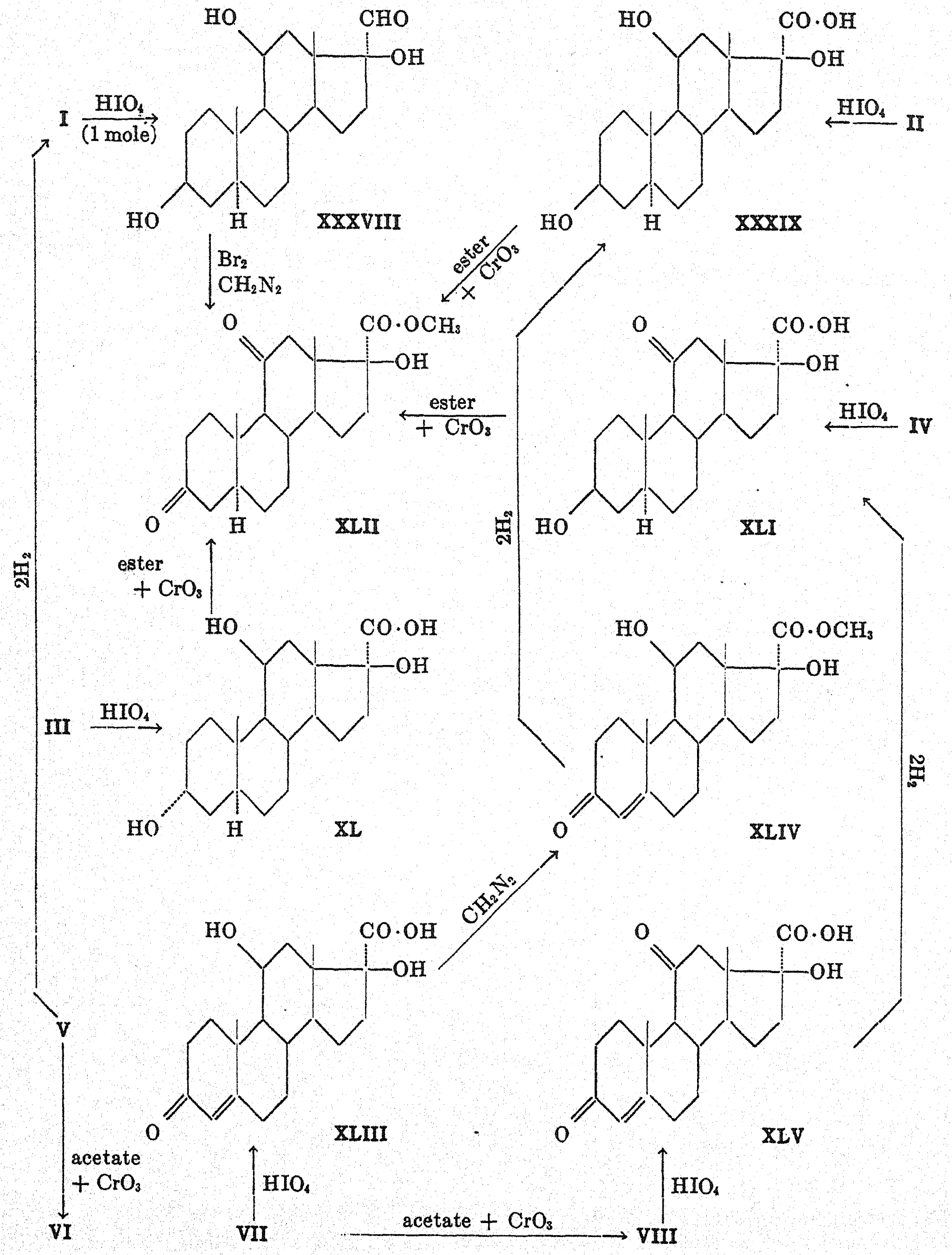
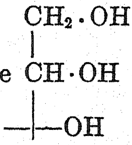
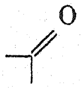
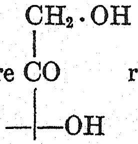
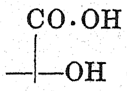
[12] Geometrical isomerism at C_{17} is not completely excluded, but is unlikely because the acids (**XXXVI, XXXVII**) are obtained by oxidative degradation of the corresponding methyl ketones, which probably possess the same (stable) configuration at C_{17}.

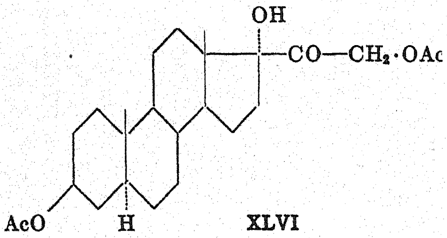

I
HIO₄ (1 mole)
HO
CHO
OH
HO
H
XXXVIII
HO
CO·OH
OH
HIO₄
II
HO
H
XXXIX
Br₂
CH₂N₂
ester + CrO₃
O
CO·OCH₃
OH
O
H
XLII
O
CO·OH
OH
ester + CrO₃
HIO₄
IV
HO
H
XLI
2H₂
2H₂
2H₂
ester + CrO₃
HO
CO·OH
OH
III
HIO₄
HO
H
XL
HO
CO·OCH₃
OH
O
XLIV
CH₂N₂
HO
CO·OH
OH
O
XLIII
O
CO·OH
OH
O
XLV
V
acetate + CrO₃
VI
HIO₄
VII
acetate + CrO₃
VIII
HIO₄

(**XXVI**), which by catalytic hydrogenation afforded (**XXIX**), whereby it is proved that the three oxygen atoms in (**XXVI**) occupy positions 3, 11 and 17. Finally, the individual members of the $C_{21}O_5$ group have been correlated both structurally and configuratively either by direct interconversion, or by indirect but unexceptionable routes, as is shown in the formulas on page 374.

In regard to the structure of the side-chain, the behavior of the individual substances toward alkaline silver solution furnishes valuable information, since α-ketols reduce this reagent immediately at 20°; aldehydes, however, give the same reaction. A final decision is possible by means of oxidation with periodic acid. Substances with the glycerol structure  $\begin{array}{c}\mathrm{CH_2\cdot OH}\\ |\\ \mathrm{CH\cdot OH}\\ |\\ -|-\mathrm{OH}\end{array}$ do not reduce alkaline silver solution at 20°, and are oxidized by excess periodic acid to ketones  containing two atoms less of carbon (113, 56, 36). Substances with the dihydroxyacetone structure  $\begin{array}{c}\mathrm{CH_2\cdot OH}\\ |\\ \mathrm{CO}\\ |\\ -|-\mathrm{OH}\end{array}$ reduce strongly, and with periodic acid yield acids  $\begin{array}{c}\mathrm{CO\cdot OH}\\ |\\ -|-\mathrm{OH}\end{array}$ containing one atom less of carbon (54). Thus (**II**) gives the acid (**XXXIX**) (39), (**III**) the acid (**XL**) (54) and (**IV**) the acid (**XLI**) (54). The methyl esters of these three acids constitute a simple means of correlating the individuals (**II**), (**III**) and (**IV**), since by oxidation with chromium trioxide they all give one and the same diketo ester (**XLII**) (39). This diketo ester was also obtained when (**I**) was oxidized partially with periodic acid, the resulting aldehyde (**XXXVIII**) converted with bromine water to the corresponding acid, and this esterified with diazomethane (39). The methyl esters have also been used to determine whether the oxygen atoms at C_3 and C_{11} are present as hydroxyl or carbonyl groups, though in some instances this has been shown by other means, *e.g.* by direct oxidation of the acetate of (**II**) to the acetate of (**IV**). In addition the methyl esters have been used to establish the configuration of hydroxyl groups in these positions (39).

The Δ^4-unsaturated compounds (**V**), (**VI**), (**VII**) and (**VIII**) have been correlated with the saturated substances; as already mentioned, (**V**) is directly convertible into (**I**), the acetate of (**V**) into that of (**VI**), and the acetate of (**VII**) into that of (**VIII**). The correlation of the two last named substances with the saturated compounds is obtained as follows: (VII) by oxidation with periodic acid gave the acid (XLIII), while (VIII) similarly afforded the acid (XLV) (54); the acid (XLIII) *via* the intermediate (XLIV) furnishes by reduction the methyl ester of (XXXIX), while the acid (XLV) by reduction and esterification yielded the methyl ester of (XLI); both (XXXIX) and (XLI) by oxidation as the esters with chromium trioxide gave the same diketo ester (XLII). It is thus proved that (V), (VI), (VII), and (VIII) possess the same configuration at C_{17} and, further, that the hydroxyl group at C_{11} in (V) and (VII) has the same configuration (β) as in (I) (39).

As mentioned above, the β-configuration of the hydroxyl group at C_3 in (I) has been established by conversion to (XXXIV); by direct correlation it can be shown that the C_3-hydroxyl groups in (II) and (IV) also possess this same β-configuration. Substance C (III) alone has a 3(α)-hydroxyl group (39). In regard to the determination of configuration at C_{17}, direct correlation with known compounds is not possible, because

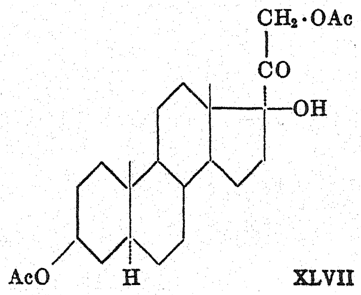


XLVI

17(α)-Hydroxy

$[\alpha]_D = -68.1°$ (dioxane)

$[M]_D = -29000°$ (")

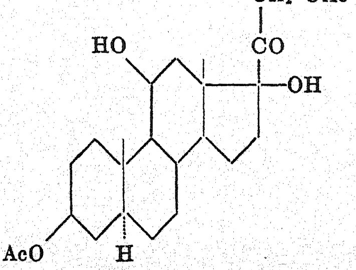


XLVII

17(β)-Hydroxy
(Diacetate of X)

$[\alpha]_D = +44.5°$ (dioxane)

$[M]_D = +19000°$ (")



Diacetate of II

no method is yet available for the removal of the oxygen atom at C_{11} without simultaneous elimination of the sensitive tertiary hydroxyl group at C_{17}. Recourse must therefore be made to optical superposition rules. The two substances, **(XLVI)** and **(XLVII)**, p. 376, with a dihydroxyacetone structure are known, and differ only in configuration at C_{17}; they show a very considerable difference in their specific rotations (72, 39).

From the large amount of available experimental data, the approximate alteration of the molecular rotation brought about by the introduction of a hydroxyl group at C_{11} can be estimated. This value is, of course, not a real constant, but for saturated *allo*pregnane derivatives amounts to about +4100° (dioxane). Addition of this value to the molecular rotations of **(XLVI)** and **(XLVII)** should give the approximate values of the molecular rotation of the diacetate of **(II)**, according as this possesses the 17(α)- or 17(β)-configuration. From the molecular rotations so calculated, the following respective specific rotations are arrived at:

$$[\alpha]_D = -56.7° \text{ (dioxane) for the } 17(\alpha)\text{-configuration}$$

$$[\alpha]_D = +52° \text{ (dioxane) for the } 17(\beta)\text{-configuration}$$

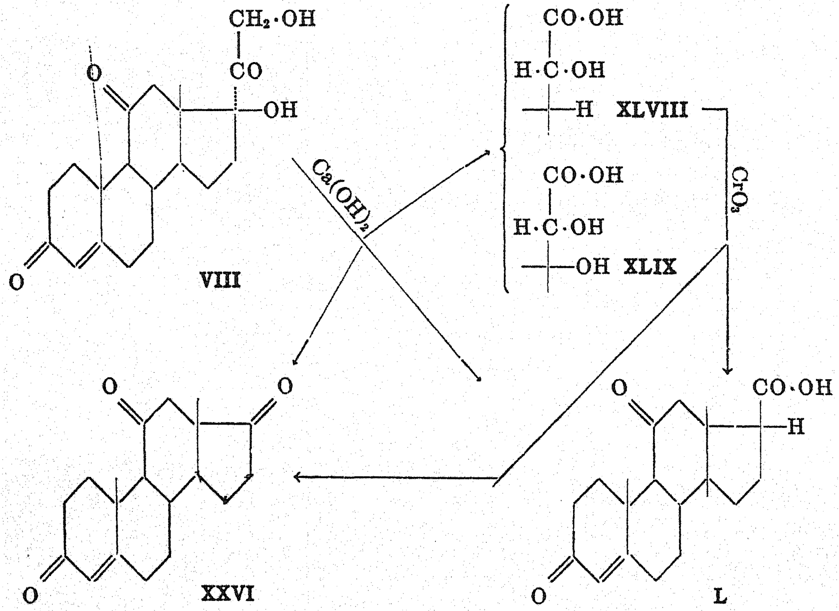
The experimental value found for the diacetate of **(II)** is $[\alpha]_D^{18} = +62.6° \pm 2°$ (dioxane), which can only be reconciled with the 17(β)-configuration (39).

In regard to the position of the double bond present in the unsaturated compounds, the following direct chemical evidence is available. The unsaturated center must be conjugated to the carbonyl group at C_3 on account of the ultraviolet-absorption spectra, hence only the Δ^1- and Δ^4-positions need be considered. That the Δ^4-position is the correct one is proved directly only for the corticosterone group (see below). The reaction sequence (given below) carried out by Mason (140) correlates, however, the unsaturated members of the $C_{21}O_5$ group and the $C_{21}O_4$ group, Sub-group *b*.

Constitution and configuration are thus established for all the members of the $C_{21}O_5$ group; only for **(III)** is the configuration of the hydroxyl group at C_{11} as yet undetermined.

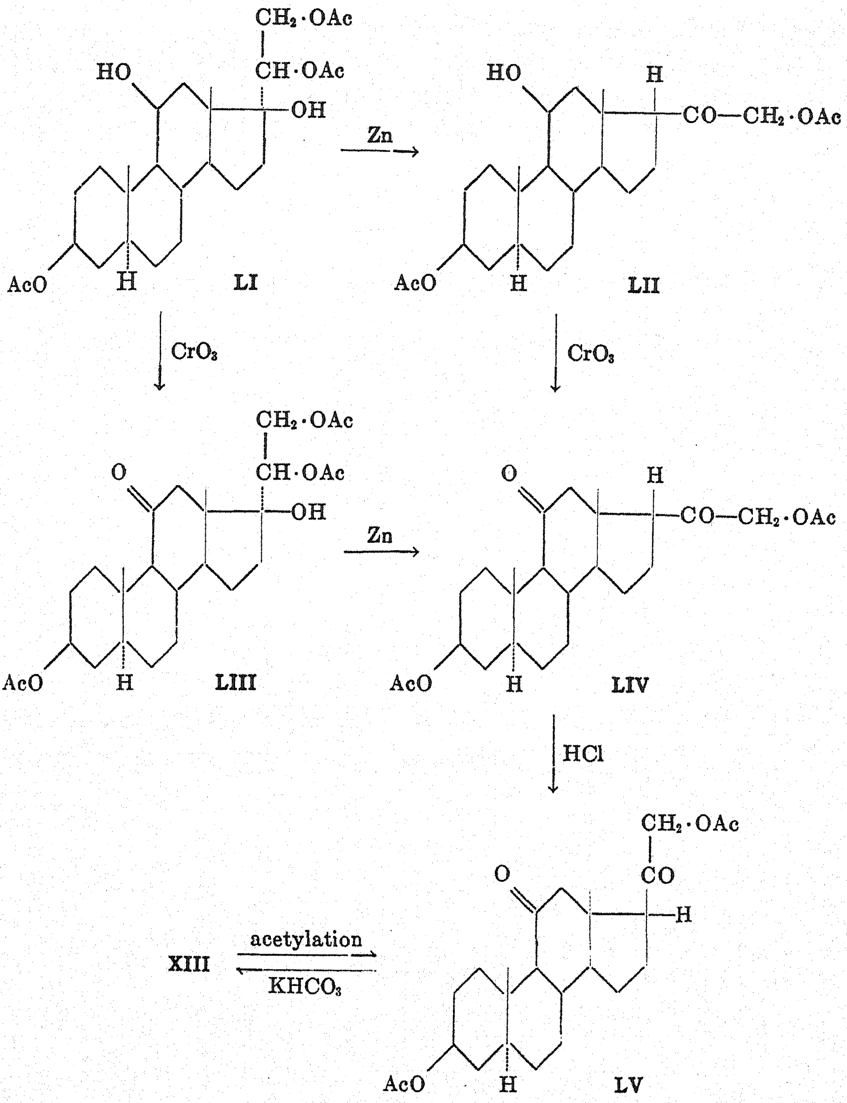
Correlation of the $C_{21}O_5$ Group with the Corticosterone Group ($C_{21}O_4$ Group, Sub-group b)

The first correlation of the $C_{21}O_5$ group with the corticosterone group (*i.e.* those members of the $C_{21}O_4$ group which contain an oxygen atom at C_{11}), was carried out by Mason (140). Treatment of **(VIII)** with aqueous calcium hydroxide yielded a mixture of acid and neutral products; from the latter **(XXVI)** was isolated. The acid fraction, which probably contained the acids (**XLVIII**) and (**XLIX**), by further oxidation with chromium trioxide gave a further quantity of (**XXVI**) together with an acid which, according to its m.p. and sp. rotation, was identical with the acid of structure (**L**) which is readily obtained from (**XV**) and (**XVI**).



Some degree of uncertainty attaches to this degradation, since 300 mg. of (**VIII**) gave only 19 mg. of acid fraction, from which 7 mg. of crude acid (**L**) were obtained. The direct conversion of a member of the $C_{21}O_5$ group to a naturally occurring member of the corticosterone group has been described by Shoppee and Reichstein (44).

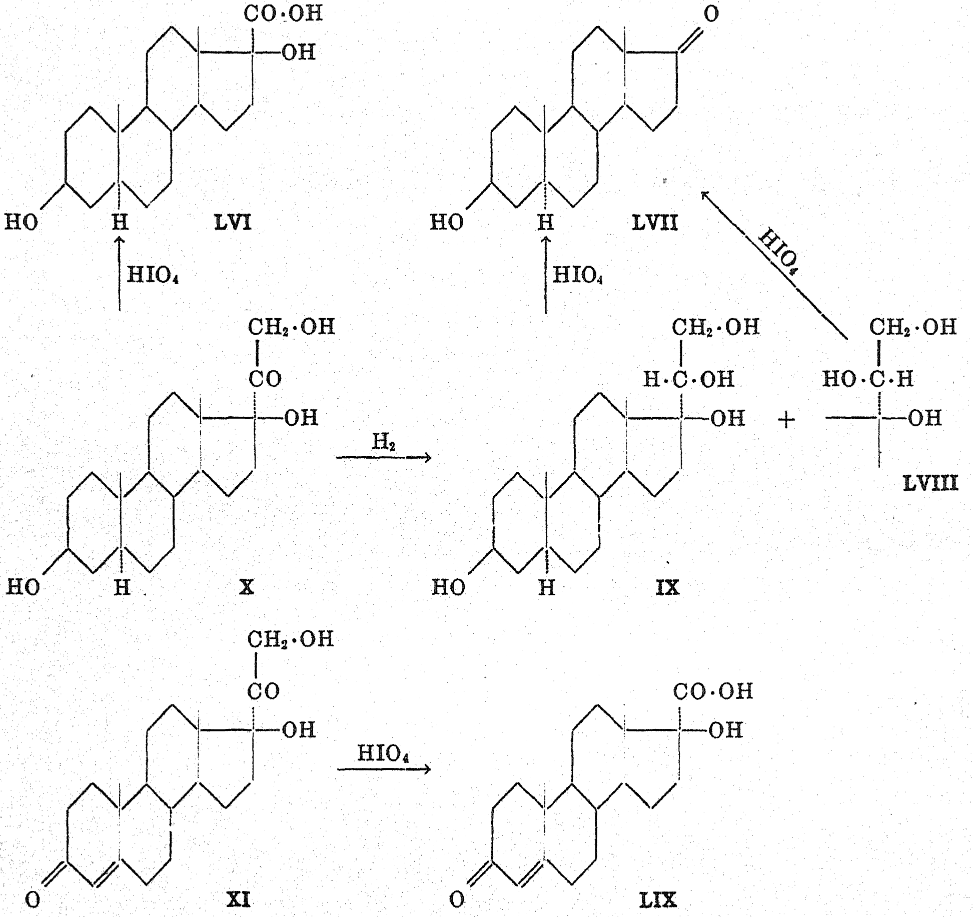
Substance A (I), as the triacetate (**LI**), was treated with zinc dust according to Miescher's (141) modification of the method of Slotta (142) (compare also Butenandt *et al.* (43), Serini *et al.* (70)) whereby one mol. of acetic acid was eliminated to give the diacetate (**LII**) of a substance which is the C_{17}-stereoisomeride of (**XII**); although (**LII**) should be the labile modification, inversion to give (**XII**) could not be achieved owing to the occurrence of simultaneous elimination of the hydroxyl group at C_{11}. Oxidation of (**LII**) yielded the diketone (**LIV**) which differs from (**LV**), the diacetate of (**XIII**), only in the configuration at C_{17}. Brief treatment of (**LIV**) with hot mineral acid furnished (**LV**), identical with the natural product, and hydrolyzed by aqueous-methanolic potassium hydrogen


CH₂·OAc
CH·OAc
HO
OH
AcO
H
LI
Zn
HO
H
CO—CH₂·OAc
AcO
H
LII
CrO₃
CrO₃
CH₂·OAc
CH·OAc
O
OH
AcO
H
LIII
Zn
O
H
CO—CH₂·OAc
AcO
H
LIV
HCl
CH₂·OAc
CO
O
H
XIII
acetylation
KHCO₃
AcO
H
LV

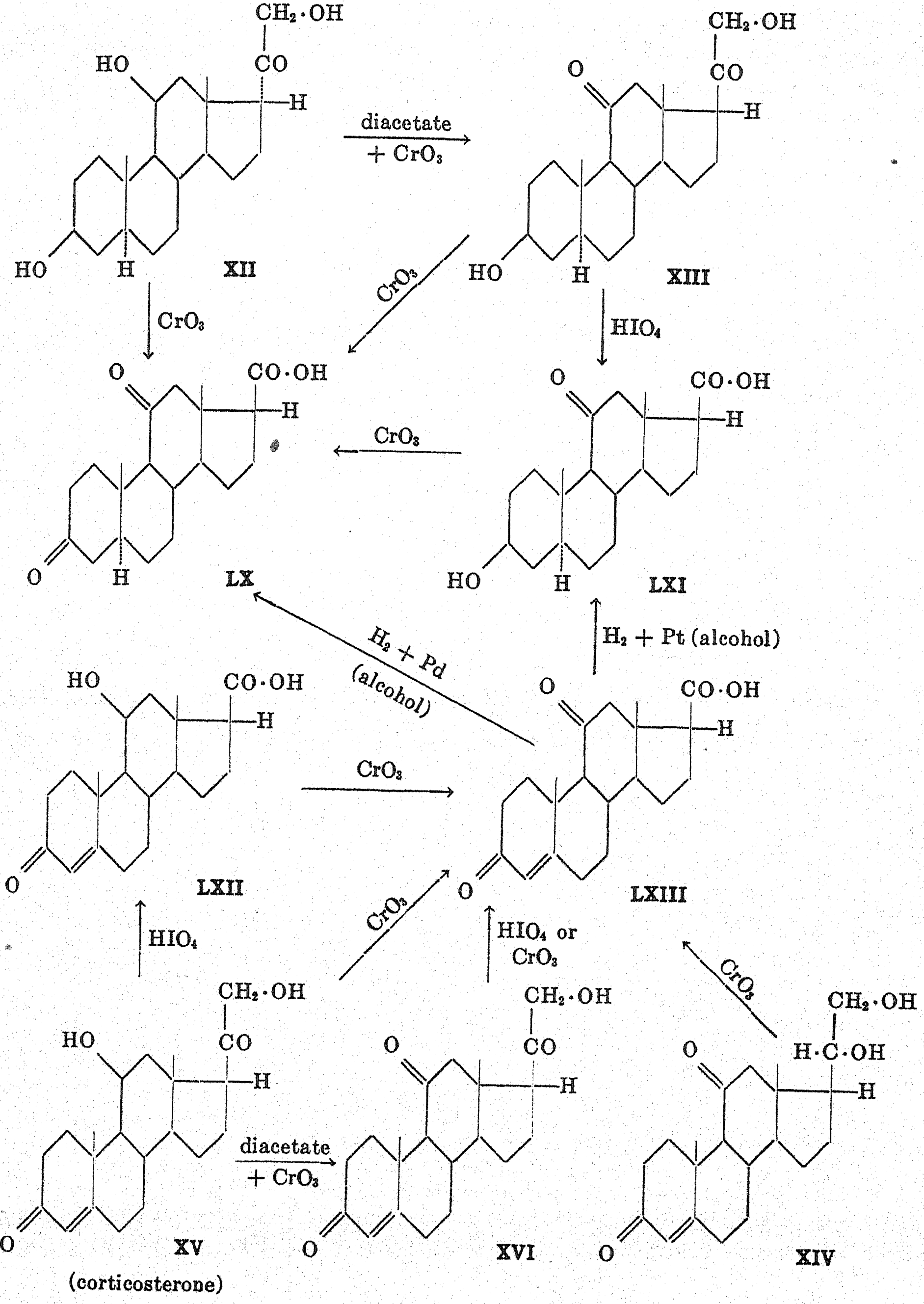
carbonate to substance N (**XIII**). An alternative route involved the prior oxidation of (**LI**) to (**LIII**), and conversion of this to (**LIV**) with zinc dust. This transformation proves the position of the side-chain in the $C_{21}O_5$-group and the configuration of the hydroxyl group at C_3 in (**XII**) and (**XIII**), since (**XII**) by oxidation as the diacetate yields (**XIII**).

2. $C_{21}O_4$ Group

Sub-group a: Without an Oxygen Atom at C_{11}. The determination of constitution and configuration for the three substances of this sub-group was relatively easy, because conversion to known substances was possible.


CO·OH
OH
HO
H
LVI
O
HO
H
LVII
HIO₄
HIO₄
HIO₄
CH₂·OH
CO
OH
HO
H
X
H₂
CH₂·OH
H·C·OH
OH
HO
H
IX
+
CH₂·OH
HO·C·H
OH
LVIII
CH₂·OH
CO
OH
O
XI
HIO₄
CO·OH
OH
O
LIX

Substance P (**X**) by degradation with periodic acid gave an acid (67) different from the known 3(β):17(α)-dihydroxy*aetioallo*cholanic acid (143), but identical with 3(β):17(β)-dihydroxy*aetioallo*cholanic acid (**LVI**) (144); with chromium trioxide (**X**) afforded the known androstane-3:17-dione. Hydrogenation of (**X**) yielded a mixture of (**IX**) and a new *allo*pregnane-3:17:20:21-tetrol (**LVIII**), both of which with periodic acid


CH₂·OH
CO
HO
H
HO
H
XII
diacetate
+ CrO₃
CH₂·OH
CO
O
H
HO
H
XIII
CrO₃
CrO₃
HIO₄
O
CO·OH
H
O
H
LX
CrO₃
O
CO·OH
H
HO
H
LXI
H₂ + Pd (alcohol)
H₂ + Pt (alcohol)
HO
CO·OH
H
O
LXII
CrO₃
O
CO·OH
H
O
LXIII
HIO₄
CrO₃
HIO₄ or CrO₃
CrO₃
CH₂·OH
CO
HO
H
O
XV
(corticosterone)
diacetate
+ CrO₃
CH₂·OH
CO
O
H
O
XVI
CH₂·OH
H·C·OH
O
H
O
XIV

furnished the known androstane-3(β)-ol-17-one (**LVII**); the nomenclature for the hydroxyl group at C_{20} is fixed by definition (66). Similarly, substance S (**XI**) by oxidation with periodic acid gave an acid different from the known 17(α)-hydroxy-3-keto-Δ^4-*aetio*cholenic acid and identical with 17(β)-hydroxy-3-keto-Δ^4-*aetio*cholenic acid (**LIX**) (68); with chromium trioxide (**XI**) furnished the known Δ^4-androstene-3:17-dione. Constitution and configuration in this sub-group are completely established by these reactions.

Sub-group b: With an Oxygen Atom at C_{11} (Corticosterone Group). The correlation of a member of this sub-group with the $C_{21}O_5$ group has already been given (see pp. 377–379). The interrelation of the five members of the sub-group among themselves has been accomplished as follows. Cautious oxidation of the acetates of (**XII**) and (**XV**) gave the acetates of (**XIII**) (73) and (**XVI**) (55) respectively. With chromium trioxide (77, 73), (**XII**) and (**XIII**) yielded the same acid (**LX**) (Kendall's "Acid 1 A"); with periodic acid, (**XIII**) furnished the acid (**LXI**) (Kendall's "Acid 1 B") which was further oxidized by chromium trioxide to give the acid (**LX**). The three unsaturated members (**XIV**), (**XV**), and (**XVI**) with chromium trioxide gave one and the same acid (**LXIII**) (77, 73, 78) (Kendall's "Acid 1") which by hydrogenation (palladium) was converted into (**LX**) or with platinum into (**LXI**). With periodic acid, (**XV**) furnished the acid (**LXII**) (77), which was converted into (**LXIII**) by chromium trioxide.

The carbon skeleton of corticosterone is established, as mentioned earlier, by its conversion into *allo*pregnane (145). The position of the oxygen atom at C_3 follows from the correlation with the $C_{21}O_5$ series; a further proof is provided by the following reaction sequence described by Shoppee and Reichstein (146) which simultaneously fixes the position of the double bond in corticosterone.

As shown by Reichstein and Fuchs (147), corticosterone by appropriate treatment with *p*-toluenesulphonyl chloride and pyridine gives a mixture of the ester (**LXIV**) and the chloroketone (**LXV**), which with sodium iodide in acetone readily yields the iodoketone (**LXVI**), reduced with extreme ease to 11-hydroxyprogesterone (**LXVII**).[13] This compound was dehydrated with hydrochloric acid to give, as the principal product, 11-dehydroprogesterone (**LXVIII**)[14] which was isolated. Hydrogenation of (**LXVIII**) and final oxidation of the hydrogenation product furnished *allo*pregnane-3:17-dione (**LXIX**) together with a little pregnane-3:17-dione (**LXX**).

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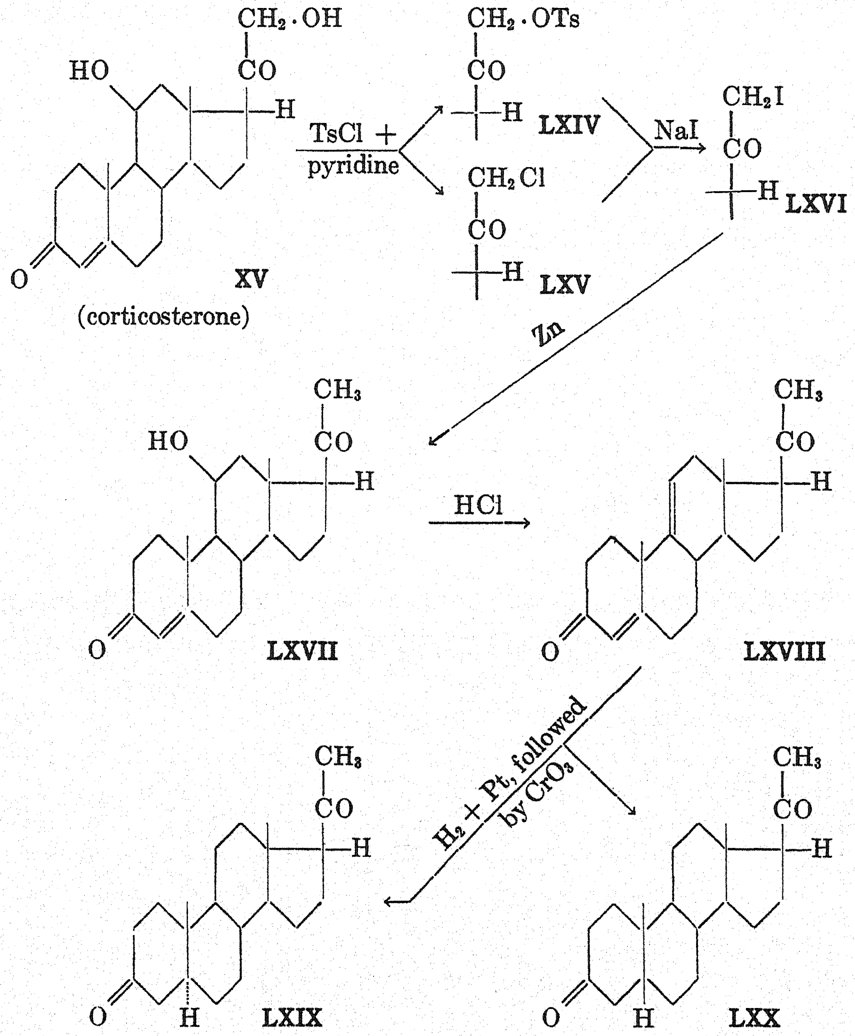
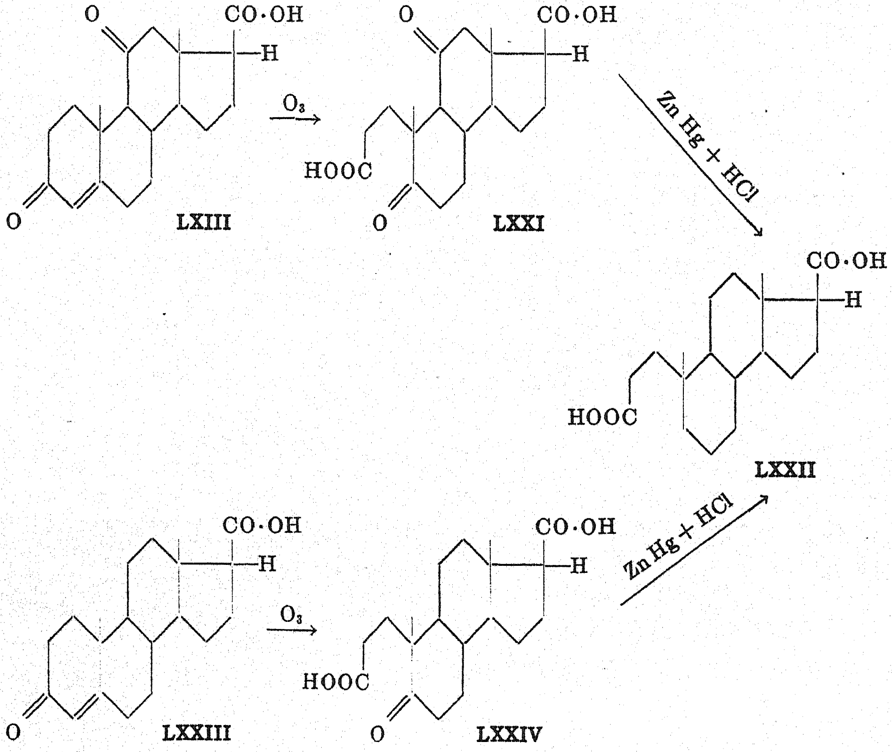
[13] In doses of 3 mg. the compound shows no progestational activity.

[14] Formulated in the original paper as Δ^{11}-unsaturated; the $\Delta^{9:11}$-formula now given is not proved but appears more probable in the light of subsequent work. The substance exhibits progestational activity approximately equal to that of progesterone itself.

This result proves the positions of the oxygen atoms at C_3 and C_{20}, and also establishes the position of the double bond. The Δ^4-position of the unsaturated center was also proved in another way (148).


CH₂·OH
CO
HO
H
O
XV
(corticosterone)
TsCl +
pyridine
CH₂·OTs
CO
H
LXIV
CH₂Cl
CO
H
LXV
NaI
CH₂I
CO
H
LXVI
Zn
CH₃
CO
HO
H
O
LXVII
HCl
CH₃
CO
H
O
LXVIII
H₂ + Pt, followed by CrO₃
CH₃
CO
H
O
H
LXIX
CH₃
CO
H
O
H
LXX

The acid (**LXIII**), obtained by oxidation of (**XV**) and (**XVI**) with chromium trioxide, was degraded as the methyl ester with ozone to a diketo-dicarboxylic acid (**LXXI**), which by Clemmensen reduction gave the dicarboxylic acid (**LXXII**). The structure of (**LXXII**) was proved by its preparation from 3-keto-Δ^4-*aetio*cholenic acid (**LXXIII**), the structure of which is known with certainty, by an analogous degradation *via* the keto-dicarboxylic acid (**LXXIV**).



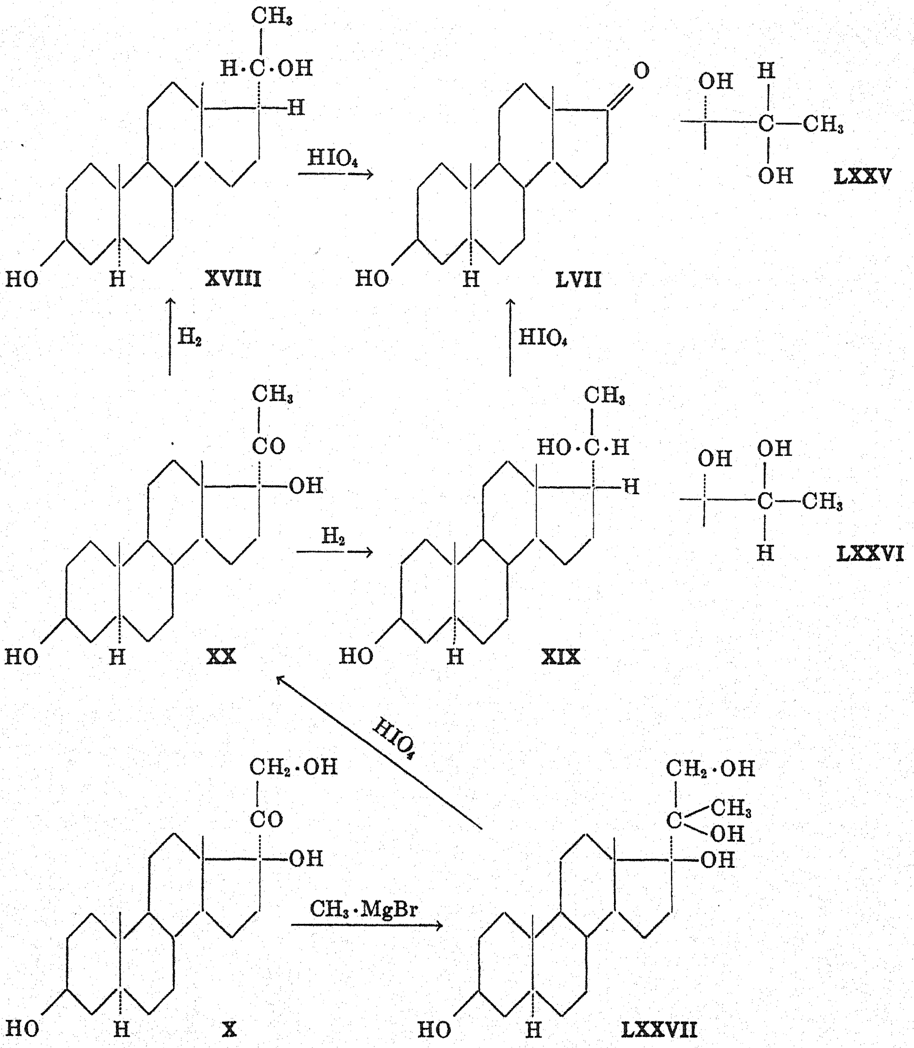
LXIII **LXXI** **LXXII** **LXXIII** **LXXIV**

The constitution and configuration of all members of the corticosterone group is thus established, apart from the stereochemical arrangement of the hydroxyl group at C_{11} in (**XII**) and (**XV**), which remains uncertain. The structure of (**XVII**) is completely unknown.

3. $C_{21}O_3$ Group

The constitution and configuration of the three substances (**XVIII**), (**XIX**), and (**XX**) follows from the facts on page 385.

Hydrogenation of (**XX**) furnished a mixture of (**XVIII**) and (**XIX**), both of which with periodic acid gave the known androstane-3(β)-ol-17-one (**LVII**). The constitutions are thus simultaneously established, while at the same time it is also proved that all three substances possess the β-configuration at C_3 and the same stereochemical arrangement at C_{17}. The compounds (**XVIII**) and (**XIX**) differ only in regard to the position of the hydroxyl group at C_{20}.


CH₃
H·C·OH
H
HO
H
XVIII
HIO₄
O
LVII
OH
H
C—CH₃
OH
LXXV
H₂
HIO₄
CH₃
CO
OH
XX
H₂
CH₃
HO·C·H
XIX
OH
OH
C—CH₃
H
LXXVI
HIO₄
CH₂·OH
CO
OH
X
CH₃·MgBr
CH₂·OH
C
CH₃
OH
OH
LXXVII

Evidence as to the configuration at C_{17} was first obtained by indirect means; an isomeride (**LXXV**), which undoubtedly has the α-configuration at C_{17}, was synthesized and found to be different from both (**XVIII**) and (**XIX**) (66). Since three isomerides differing only in stereochemical arrangement at C_{20} are not possible, (**XVIII**) and (**XIX**) must possess the β-configuration at C_{17}. The second theoretically possible isomeride (**LXXVI**) of the 17α-series was known (87), but its method of preparation was such as to preclude any deductions as to its configuration at C_{17}.

Later, the configuration at C_{17} of (**XVIII**), (**XIX**), and (**XX**) was proved directly as follows (89): (X), whose configuration is completely established, was treated with methyl magnesium bromide and the crude reaction product (**LXXVII**) (probably a mixture of two isomerides) degraded with periodic acid to give (**XX**).

The constitution of 17(β)-hydroxyprogesterone (**XXI**) was proved by degradation (92, 93), and the configuration by partial synthesis (91, 90).

The constitution of desoxycorticosterone (**XXII**) called for no special proof, because the substance had been prepared by partial synthesis prior to its isolation from the gland. Its conversion to progesterone (148) has been described.

4. $C_{21}O_2$ Group and Steroids with Less Than 21 Carbon Atoms

The constitutions of (**XXIII**), (**XXIV**), (**XXVII**) and (**XXVIII**) were known before the substances were isolated from the adrenal cortex. The determination of the constitutions of (**XXV**) and (**XXVI**) has already been dealt with in connection with the $C_{21}O_5$ group.

IV. PARTIAL SYNTHESIS OF THE NATURAL AND RELATED STEROIDS

Since the total synthesis of the carbon skeleton characteristic of the steroids has not yet been accomplished, the synthetic preparation of substances isolated from the adrenal cortex and of related compounds must start from sterols, bile acids, sapogenins, or other natural products which already contain the finished 4-ring system. Further, natural sterols or steroids containing an oxygen atom at C_{11} (apart from those of the adrenal cortex) are not known with certainty[15] and the synthetic introduction of an oxygen atom into the steroid molecule at C_{11} has not yet been accomplished.[16] Hence none of the substances isolated from the adrenal cortex containing an oxygen atom at C_{11}, that is, the whole $C_{21}O_5$ group and the corticosterone group ($C_{21}O_4$ group; Sub-group *b*) are yet available synthetically. All the other substances can be prepared.

1. $C_{21}O_4$ Group, Sub-group a, and Related Compounds

17-Ethinylandrostane-3(β):17(α)-diol (**LXXVIII**) (150, 151, 152), obtained from androstane-3(β)-ol-17-one (**LVII**) by condensation with

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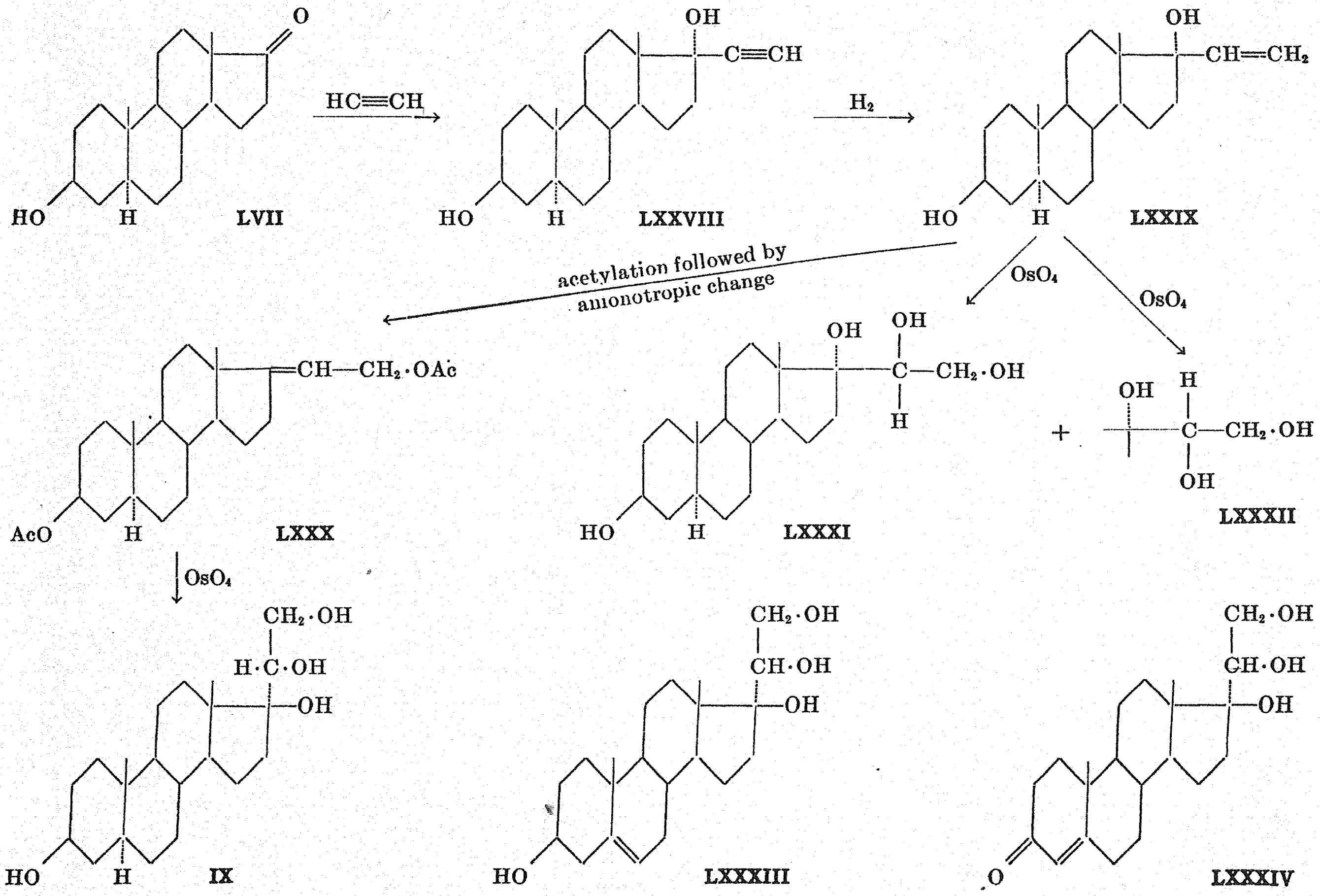
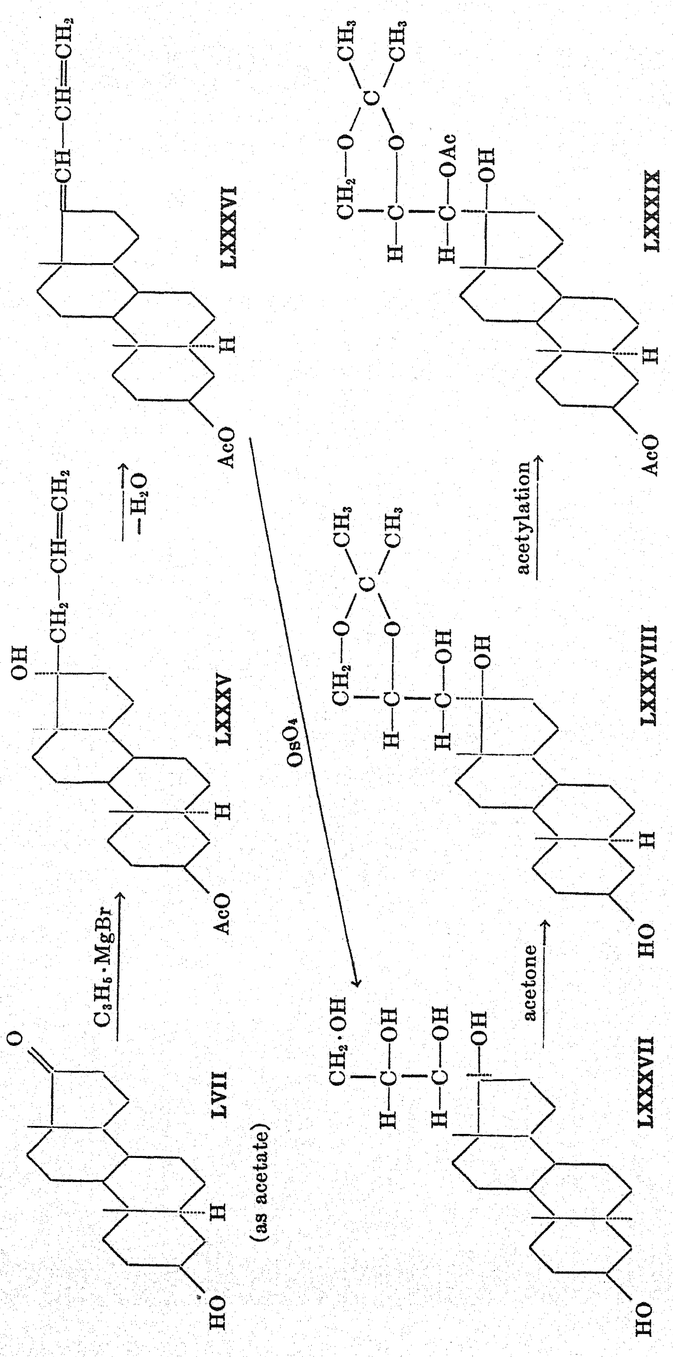
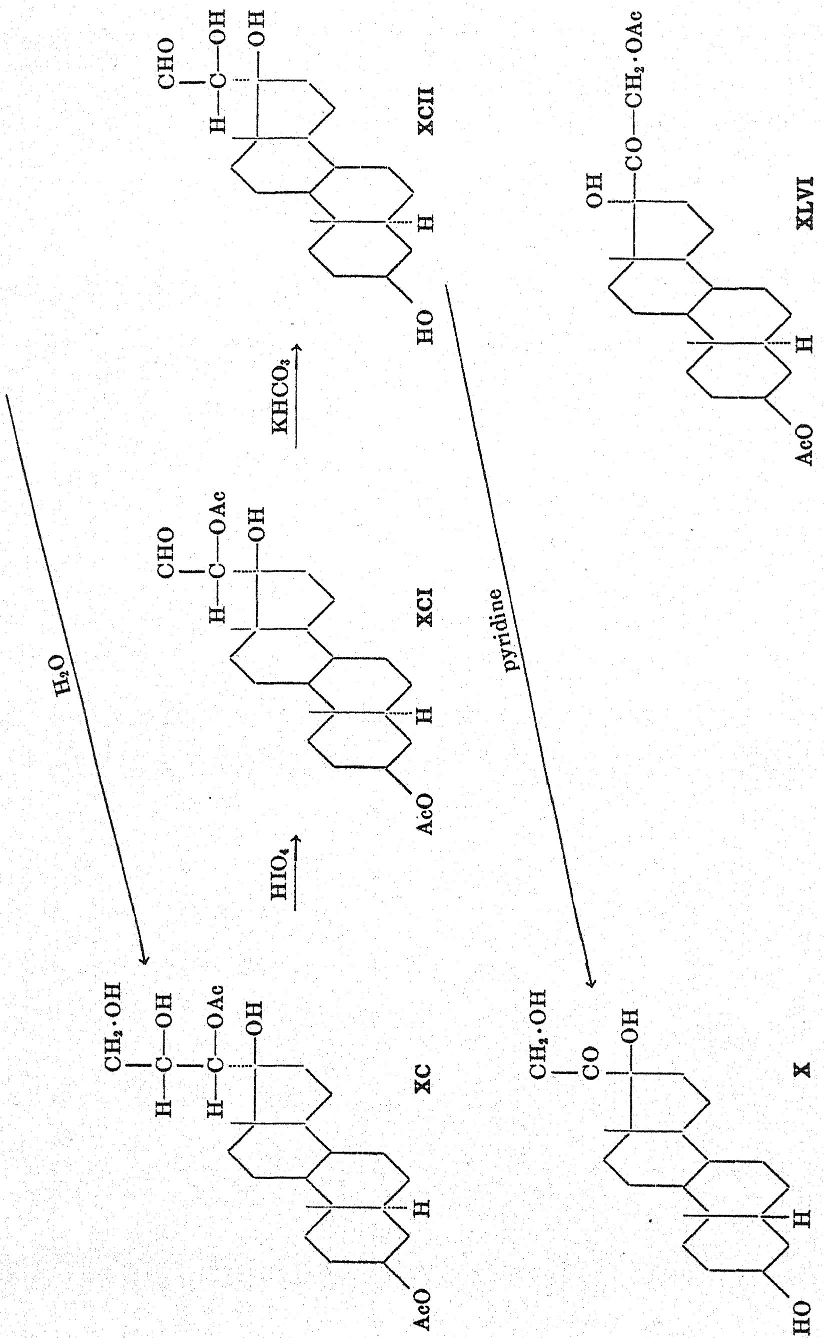
[15] Sarmentogenin possibly contains a hydroxyl group at C_{11} (131), but this is very uncertain and the compound is extremely rare. A formula for ouabain containing a hydroxyl group at C_{11} has been proposed (149), but this is equally uncertain.

[16] Wieland and Posternak (123) and Wieland and Dane (124) have prepared 11-hydroxy-12-ketocholanic acid and 11:12-diketocholanic acid, and also the 3:11-dihydroxy-12-ketocholanic acid is known (125, 128, 129), but elimination of the unwanted carbonyl group at C_{12} has not yet been effected (126, 127, 125, 128, 129).



LVII
HC≡CH
LXXVIII
H₂
LXXIX
acetylation followed by anionotropic change
OsO₄
OsO₄
LXXX
LXXXI
+
LXXXII
OsO₄
IX
LXXXIII
LXXXIV


LVII
(as acetate)
$C_3H_5 \cdot MgBr$
LXXXV
$-H_2O$
LXXXVI
OsO_4
LXXXVII
acetone
LXXXVIII
acetylation
LXXXIX
HO
AcO
H
O
OH
CH_2
CH
$CH_2 \cdot OH$
CH_3
OAc


CH₂·OH
H—C—OH
H—C—OAc
OH
H
AcO
H₂O
HIO₄
CHO
H—C—OAc
OH
H
AcO
KHCO₃
CHO
H—C—OH
OH
H
HO
pyridine
CH₂·OH
CO
OH
H
HO
OH
CO—CH₂·OAc
H
AcO

XC

XCI

XCII

X

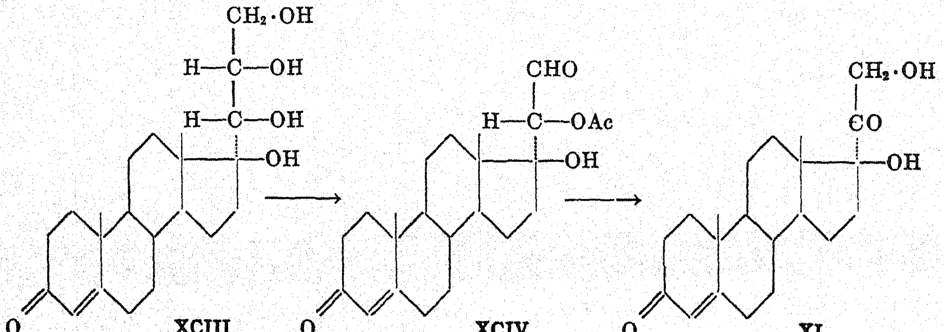
XLVI

acetylene (Nef's reaction (51)) by partial hydrogenation gave 17-vinyl-androstane-3(β):17(α)-diol (**LXXIX**), hydroxylated by Criegee's method (153) with osmium tetroxide to the two isomeric *allo*pregnane-tetrols (**LXXXI, LXXXII**) (154, 70).

Anionotropic change of the 3:17-diacetate of (**LXXIX**) to the 3:21-diacetate (**LXXX**) (69, 70) and hydroxylation of this gave as the principal product (**IX**) identical with the natural substance K (69).

The two Δ^5-tetrols (154, 155) corresponding stereochemically with (**LXXXI**) and (**LXXXII**), the two Δ^4-3-keto-17(α)-triols (**CXIX, CXX**) (p. 395) (154, 155), the Δ^5-17(β)-tetrol (**LXXXIII**) (156), and the Δ^4-3-keto-17(β)-triol (**LXXXIV**) (157, 158) have been prepared similarly. In the two last-named compounds the configuration at C_{20} is undetermined.

Great difficulty attended the partial synthesis of Substance P (**X**) and Substance S (**XI**). In the case of (**X**) (71), the acetate of androstane-3(β)-ol-17-one (**LVII**) with allyl magnesium bromide gave the compound (**LXXXV**), dehydrated to the diene (**LXXXVI**), which by hydroxylation with osmium tetroxide gave, besides an isomeride and other products, the pentol (**LXXXVII**). The synthesis up to this point follows the route first described by Butenandt and Peters (159) for the preparation of the unsaturated keto-tetrol (**XCIII**) (see below), the configuration of the various substances formed being established with some certainty. The pentol (**LXXXVII**) was condensed with acetone by use of anhydrous copper sulphate and the acetone derivative (**LXXXVIII**) converted to the diacetate (**LXXXIX**). Cautious hydrolysis removed acetone to furnish the 3:20-diacetate (**XC**), which was oxidized with periodic acid to the diacetoxyaldehyde (**XCI**). Gentle hydrolysis afforded the free trihydroxyaldehyde (**XCII**) which was rearranged by boiling pyridine to yield (**X**) identical with the natural product. A synthesis of the 17-epimeride and its diacetate (**XLVI**) (already mentioned on p. 376) has also been described (72); Δ^5-pregnene-3(β):17(β):21-triol-20-one, which corresponds with (**X**) in configuration but also possesses Δ^5-unsaturation, has also been prepared (160).



XCIII **XCIV** **XI**

Substance S (**XI**) has been obtained in an analogous manner (74) from the tetrol (**XCIII**), described by Butenandt and Peters (159), by way of the acetylated aldehyde (**XCIV**) (161). A simpler method for the direct degradation of (**XCIII**) to the free aldehyde corresponding to (**XCIV**) with periodic acid has also been described (75).

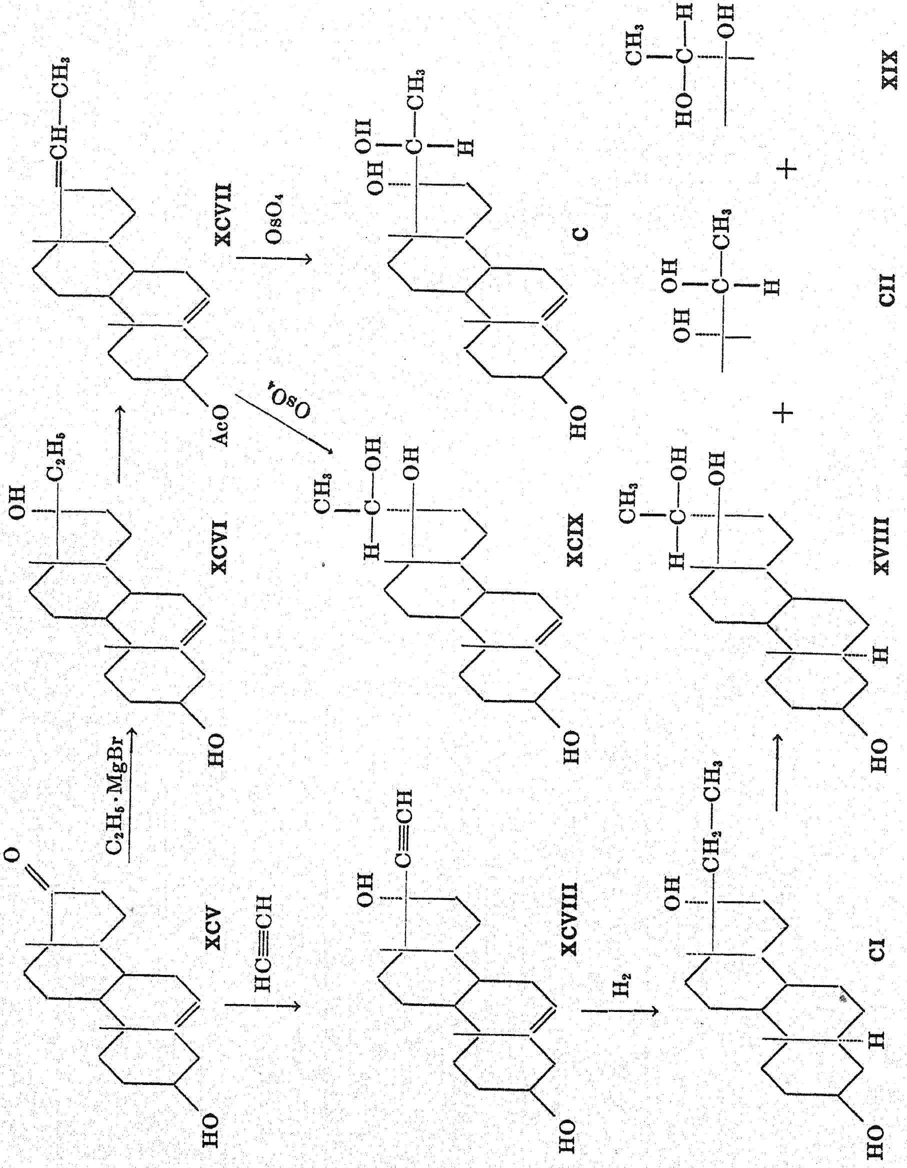
The bioassay of the aldehyde (**XCIV**) is of some interest; preliminary work shows that the compound is at least markedly less active than desoxycorticosterone acetate in the Everse-de Fremery test (162), and a provisional examination by Long showed that with 0.5 mg. it had no effect on carbohydrate metabolism (no increase in liver glycogen) in mice (163).

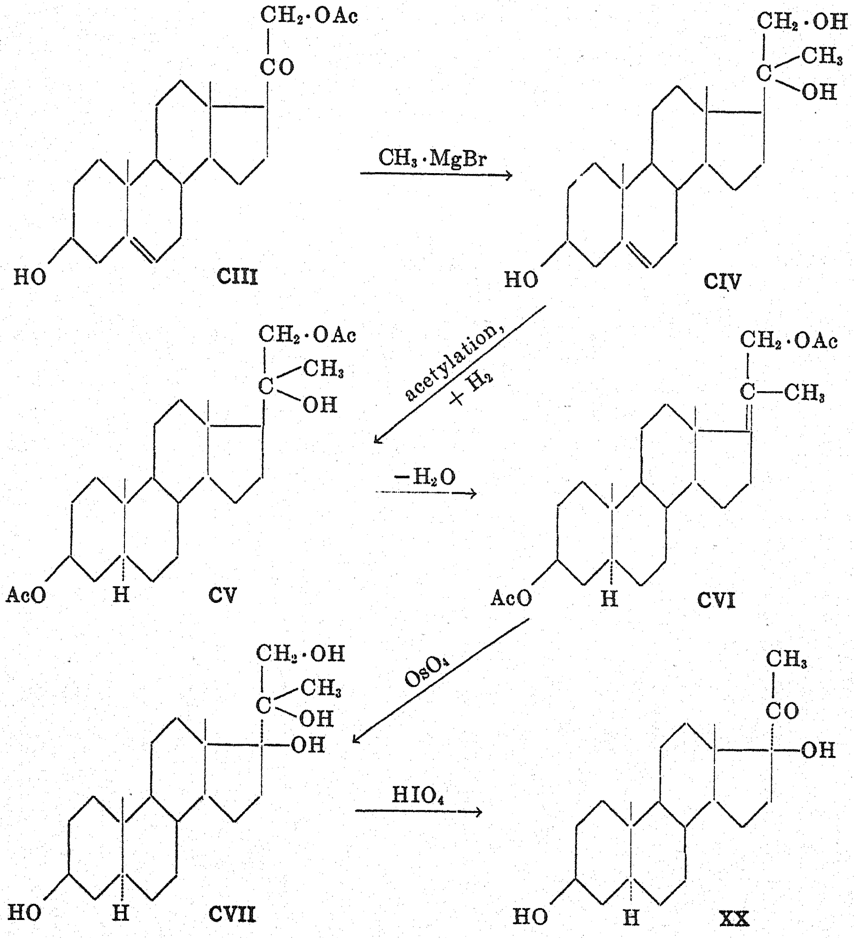
2. $C_{21}O_3$ Group and Related Compounds

The two unsaturated triols (**XCIX, C**) were synthesized by Butenandt *et al.* (43) as shown on page 392.

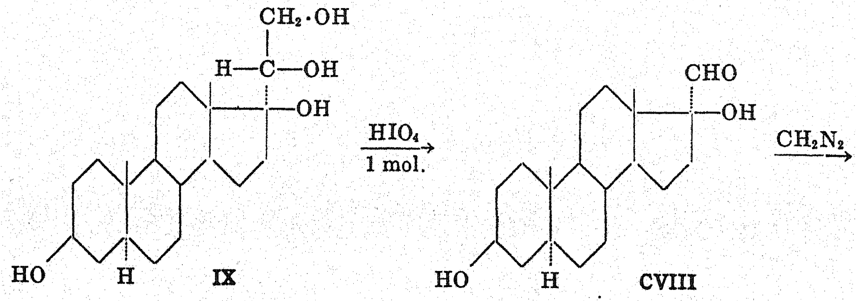
Δ^5-Androstene-3(β)-ol-17-one (**XCV**) by treatment with ethyl magnesium bromide gave Δ^5-pregnene-3(β):17(α)-diol (**XCVI**); for configuration see (144). Acetylation of (**XCVI**) followed by dehydration furnished the acetate of $\Delta^{5:17}$-pregnadiene-3(β)-ol (**XCVII**), which was hydroxylated with osmium tetroxide to yield the two isomeric Δ^5-pregnene-3(β):17:20-triols (**XCIX, C**); for configuration see (66). Similarly, from the saturated *allo*pregnane-3(β):17(α)-diol (**CI**)—which is most readily accessible by hydrogenation of (**XCVIII**)—are obtained the two saturated 3(β):17:20-triols (**XVIII, CII**), of which (**XVIII**) is identical with natural substance J (86, 87). The isomeric triol (**XIX**) was also isolated in very small quantity, and shown to be identical with the natural substance O. The fourth isomeric triol of the 17(α)-series has also been prepared, but in another way (66).

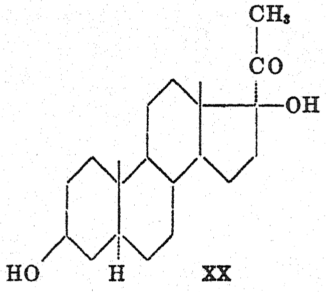
The synthesis of (**XX**) presented more difficulty; as already mentioned (p. 386), (**XX**) can be prepared from (**X**) (89), but two further methods have been described. The 21-monoacetate of Δ^5-pregnene-3(β):21-diol-20-one (**CIII**), an intermediate in the synthesis of desoxycorticosterone (**XXII**), was treated with methyl magnesium bromide, whereby a mixture of two C_{20}-stereoisomeric triols (**CIV**) were formed. These were separated as the acetates and hydrogenated to the corresponding saturated triol diacetates (**CV**). The (**CV**) obtained in larger quantity was dehydrated to (**CVI**), and the mixture of tetrols (**CVII**) obtained by hydroxylation of (**CVI**) degraded with periodic acid to give (**XX**), identical with natural substance L (90).


XCV
C₂H₅·MgBr
XCVI
XCVII
AcO
OsO₄
C
HC≡CH
XCVIII
OsO₄
XCIX
H₂
CI
XVIII
CII
XIX
+
+


CH₂·OAc
CO
HO
CIII
CH₃·MgBr
CH₂·OH
CH₃
C
OH
HO
CIV
acetylation,
+ H₂
CH₂·OAc
CH₃
C
OH
AcO
H
CV
−H₂O
CH₂·OAc
C—CH₃
AcO
H
CVI
OsO₄
CH₂·OH
CH₃
C
OH
—OH
HO
H
CVII
HIO₄
CH₃
CO
—OH
HO
H
XX

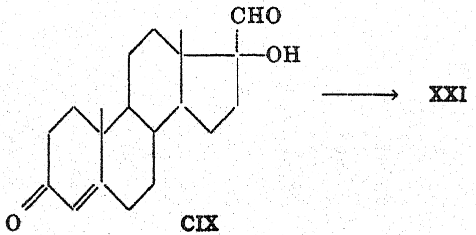
The second method (91) is simpler and gives additional proof for the configuration at C_{17}. Careful oxidation of (IX) with periodic acid (1 mol.)


CH₂·OH
H—C—OH
—OH
HO
H
IX
HIO₄
1 mol.
CHO
—OH
HO
H
CVIII
CH₂N₂

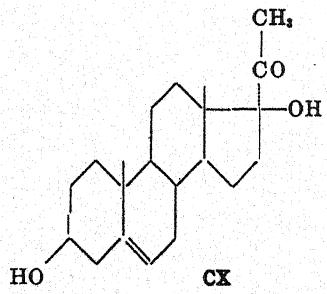


XX

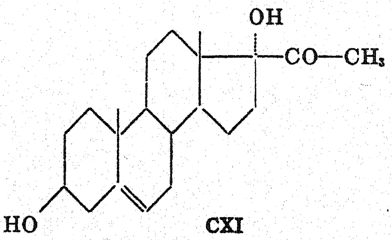
furnished the aldehyde (**CVIII**), converted to (**XX**) in moderate yield by diazomethane. Similarly, the ketotriol (**LXXXIV**) has been converted *via* the aldehyde (**CIX**) into 17(β)-hydroxyprogesterone (**XXI**) (90, 91); Δ^5-pregnene-3(β):17(β)-diol-20-one (**CX**) has been prepared (91, 160), and the 17(α)-stereoisomeride (**CXI**) likewise obtained (164, 165).



CIX

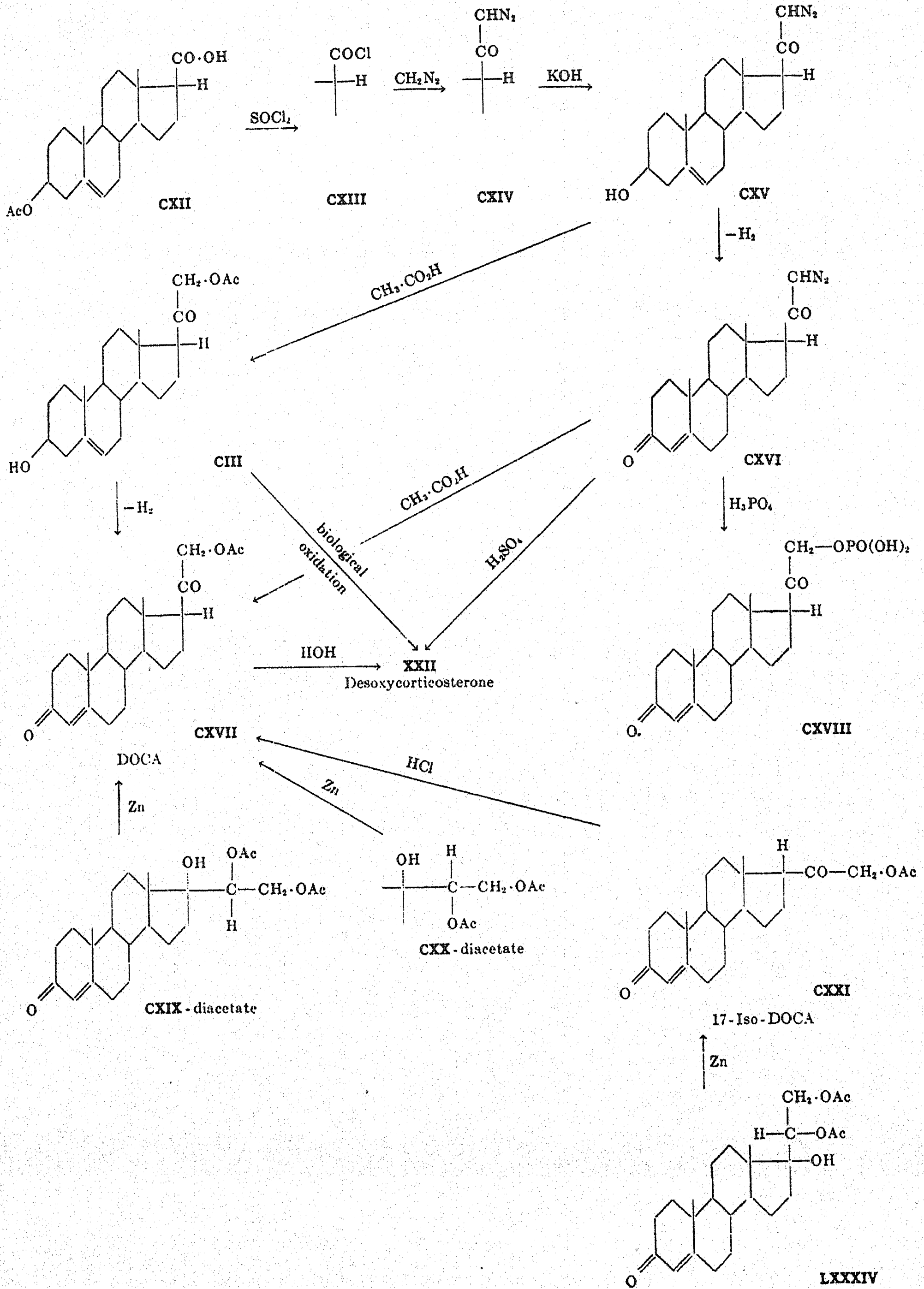


CX



CXI

The 17(α)-hydroxyketones appear to be more sensitive to alkaline reagents than the 17(β)-isomerides (165, 94), but both series of compounds undergo intramolecular rearrangement to perhydrochrysene:D-*homo*-

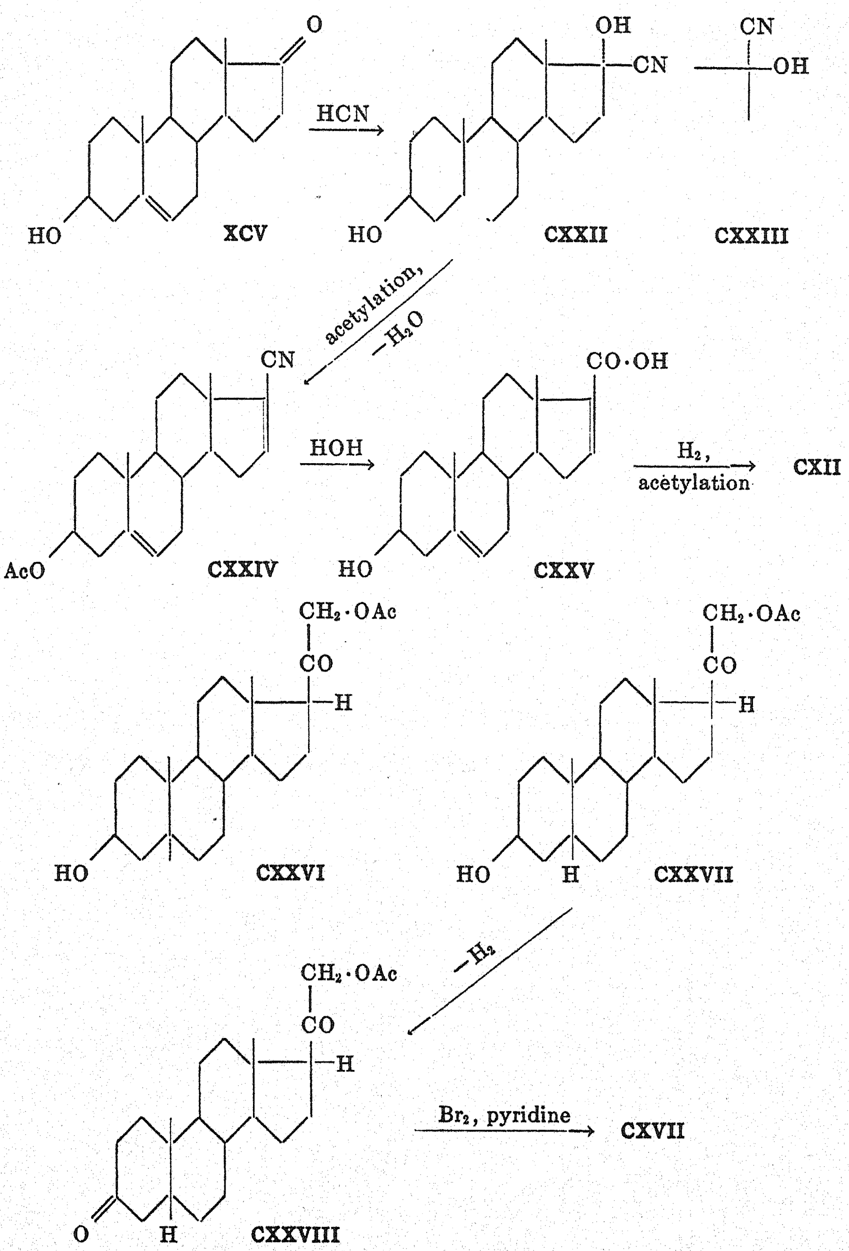

CO·OH
H
AcO
CXII
SOCl₂
COCl
H
CXIII
CH₂N₂
CHN₂
CO
H
CXIV
KOH
CHN₂
CO
H
HO
CXV
−H₂
CH₂·OAc
CO
H
HO
CH₃·CO₂H
CHN₂
CO
H
O
CXVI
CIII
CH₃·CO₂H
biological oxidation
H₂SO₄
−H₂
H₃PO₄
CH₂·OAc
CO
H
O
CXVII
DOCA
HOH
XXII
Desoxycorticosterone
CH₂—OPO(OH)₂
CO
H
O
CXVIII
HCl
Zn
Zn
OH
OAc
C—CH₂·OAc
H
O
CXIX - diacetate
OH
H
C—CH₂·OAc
OAc
CXX - diacetate
H
CO—CH₂·OAc
O
CXXI
17 - Iso - DOCA
Zn
CH₂·OAc
H—C—OAc
OH
O
LXXXIV

androstane (166), chrysoallopregnane (164) derivatives. Rearrangement also occurs by treatment with acids or simply by heating (94).

The partial synthesis of desoxycorticosterone (**XXII**) from 3(β)-acetoxy-Δ^5-*aetio*cholenic acid (**CXII**) was described as early as 1937 (95). The acetoxyacid (**CXII**) was converted by way of the acid chloride (**CXIII**) and the acetoxydiazoketone (**CXIV**) into the free hydroxydiazoketone (**CXV**); this by warming with acetic acid gave the 21-acetate (**CIII**), oxidized by chromium trioxide (after protection of the Δ^5-double bond) or by the Oppenauer method (167, 168) to (**CXVII**), hydrolysis of which with acids or potassium hydrogen carbonate furnished (**XXII**). Alternatively, (**CXV**) was converted by oxidation to diazoprogesterone (169) (**CXVI**), which by treatment with acetic acid afforded (**CXVII**) or with dilute sulphuric acid yielded (**XXII**) directly. The phosphoric acid ester (**CXVIII**) of (**XXII**), which cannot be prepared directly from (**XXII**), was obtained (170) from diazoprogesterone (**CXVI**); the *p*-toluenesulphonic ester of (**XXII**), which is extremely reactive by reason of the activating influence of the carbonyl group at C_{20}, has also been prepared (170) from diazoprogesterone (**CXVI**). Other organic esters can be prepared directly from (**XXII**) without difficulty. The oxidation of (**CIII**) with a microorganism described as "*Coryne-bact. mediolanum*" has been performed by Mamoli (171, 15), simultaneous hydrolysis of the 21-acetoxy group occurring to give (**XXII**) directly.

The phosphoric acid ester (**CXVIII**) was found to be only about as active as free desoxycorticosterone (**XXII**) in the Everse-de Fremery test (170), and it possesses little or no activity in Ingle's test (61). A noteworthy result has been reported by Selye (172), according to which 3-hydroxy-21-acetoxy-Δ^5-pregnene-20-one (**CIII**) was about as active as (**XXII**) in survival tests in rats; some activity of this compound (**CIII**) in dogs has been reported previously (97), whereas it proved to be practically inactive in the Everse-de Fremery test (2).

The acid (**CXII**) required for the synthesis of (**CXVII**) can be prepared by direct degradation of sterols (173), indirectly from sapogenins (174), and has also been built up from Δ^5-androstene-3(β)-ol-17-one (**XCV**) by way of the cyanohydrins (**CXXII, CXXIII**), the doubly unsaturated nitrile (**CXXIV**), and the $\Delta^{5:16}$-unsaturated acid (**CXXV**) (175). In a similar way have been prepared the stereoisomeric compounds (**CXXVI**) (176) and (**CXXVII**) (177); the latter has been converted *via* the diketone (**CXXVIII**) into (**CXVII**).


O
HO
HCN
OH
CN
HO
CN
OH
acetylation,
$-H_2O$
CN
AcO
HOH
CO·OH
HO
H_2,
acetylation
CXII
$CH_2·OAc$
CO
H
HO
$CH_2·OAc$
CO
H
HO
H
$-H_2$
$CH_2·OAc$
CO
H
O
H
Br_2, pyridine
CXVII

XCV **CXXII** **CXXIII**

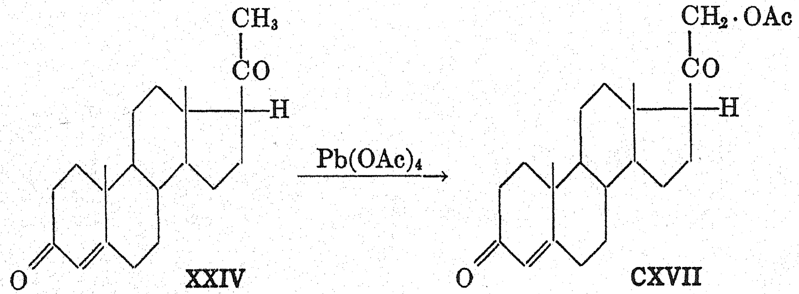
CXXIV **CXXV**

CXXVI **CXXVII**

CXXVIII

An essentially different approach to the preparation of (**XXII**) has been described by Serini *et al.* (70); these authors heated the 20:21-diacetate of one of the two isomeric Δ^4-pregnene-17(α):20:21-triol-3-ones (**CXIX, CXX**) (or the mixture of both C_{20} isomerides) with zinc dust according to the method of Slŏtta (142) (cf. Butenandt *et al.*, 43), whereby (**CXVII**) was obtained directly. Using the same procedure but starting from the 20:21-diacetate of the 17(α)-isomeride (**LXXXIV**), Shoppee (45) prepared the labile 17-*iso*desoxycorticosterone acetate (**CXXI**), whose configuration at C_{17} is the inverse of that of (**CXVII**); the compound (**CXXI**) was inactive in the survival test in rats, but readily underwent inversion in the presence of hot dilute hydrochloric acid to give the biologically active stable isomeride (**CXVII**).

Ehrhart *et al.* (178) found that progesterone (**XXIV**) could be oxidized directly to desoxycorticosterone acetate (**CXVII**) with lead tetracetate according to the method of Dimroth and Schweizer (179); the yield in this particular case is, however, so poor (180, 181) that it is of no practical value for the preparation of (**CXVII**).

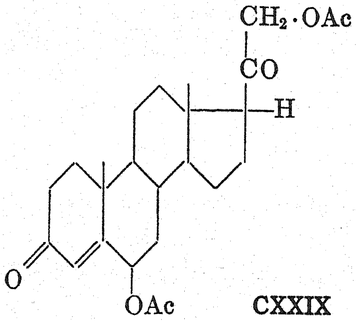


XXIV Progesterone

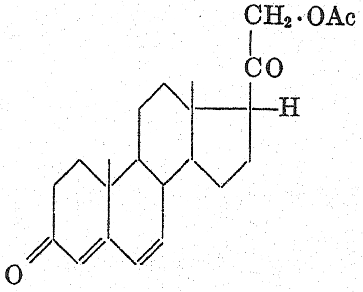
CXVII DOCA

Another method for the preparation of (**CXVII**) starting from sarsasapogenin, and involving the oxidation of 4-bromopregnane-3:20-dione with persulphuric acid, has been indicated by Marker (182).

The preparation of 6-hydroxydesoxycorticosterone diacetate (6-acetoxy-DOCA) (**CXXIX**) has been described by Ehrenstein (183); in Ingle's test (184) it was found to possess no influence upon the work performance of adrenalectomized rats in doses of up to 2 mg. per day, but in large dosage it enabled rats to gain in weight following adrenalectomy. Wettstein (185) by a variation of Oppenauer's method (167), using quinone and aluminum *tert.* butoxide, found a new and useful procedure for the preparation of doubly unsaturated ketones. Starting from (**CIII**) he obtained 6-dehydrodesoxycorticosterone-acetate (**CXXX**); this compound proved to be inactive at a dosage level of 1 mg. per day in the survival test in rats (under similar conditions 0.25 mg. DOCA (**CXVII**) gave full response). This result is remarkable as 6-dehydroprogesterone, prepared by the same method, had about 50 per cent of the progestational activity of progesterone (**XXIV**) (185).

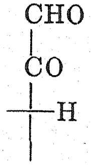

CH₂·OAc
CO
H
O
OAc

CXXIX

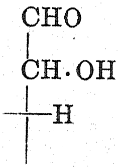

CH₂·OAc
CO
H
O

CXXX

The partial synthesis of ketoaldehydes and hydroxyaldehydes of the types:

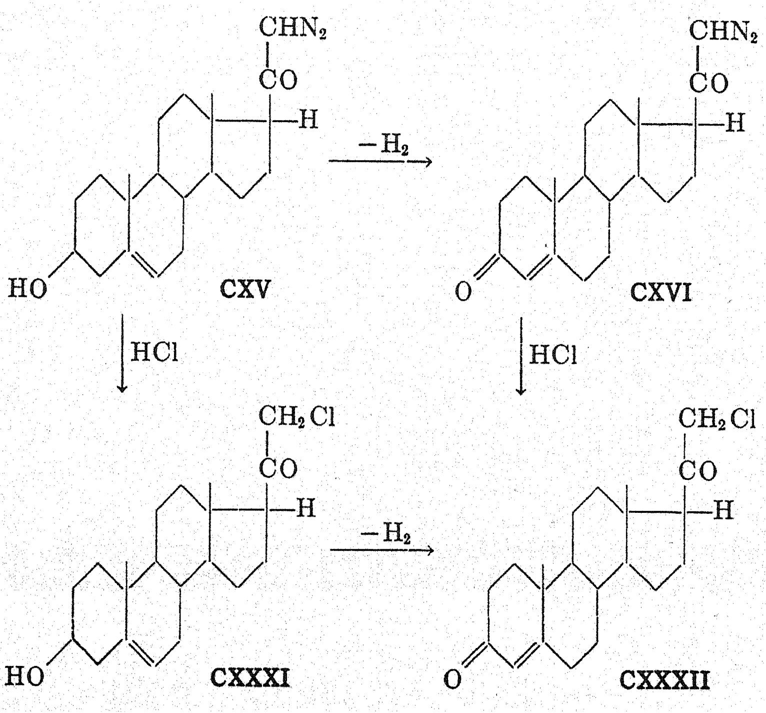


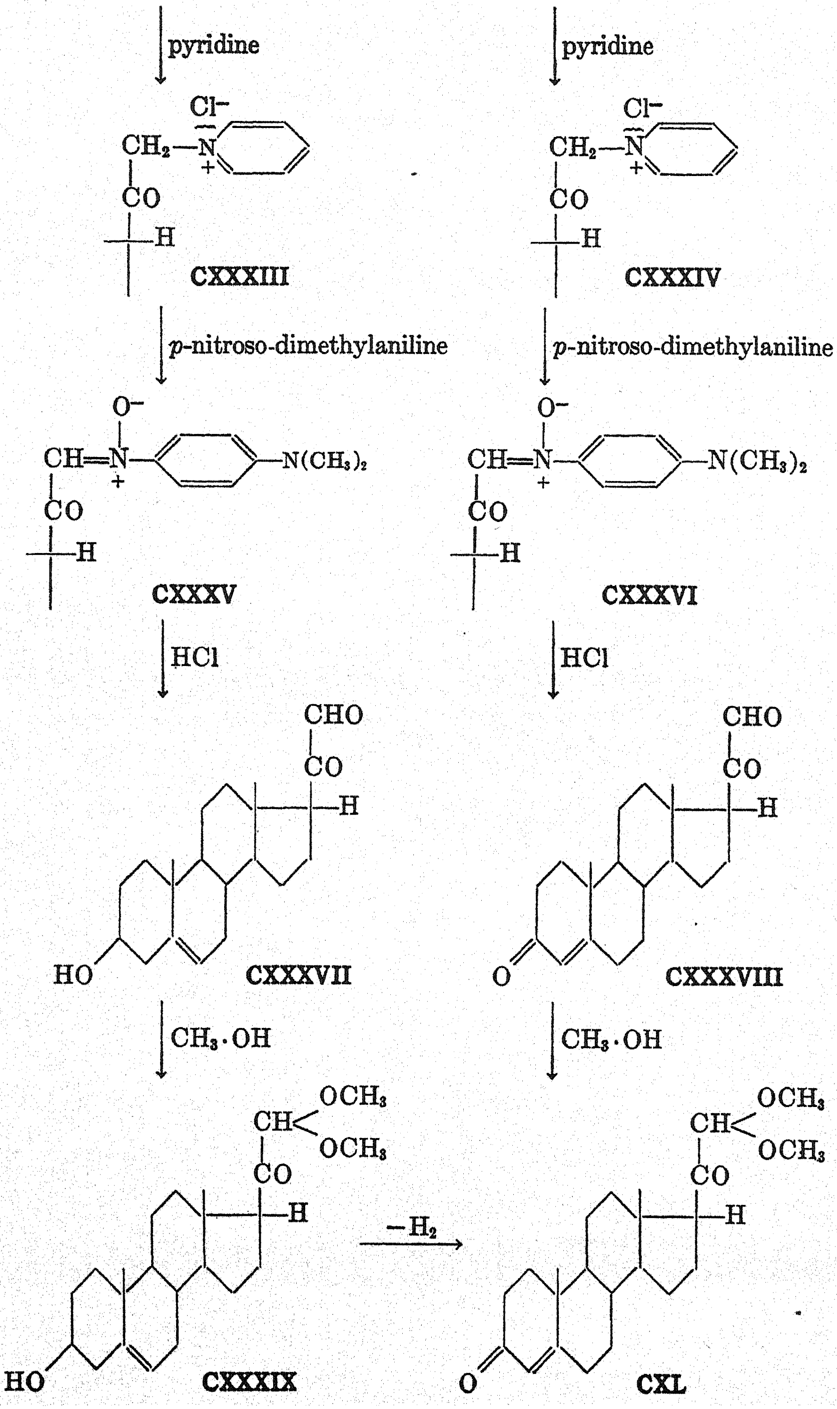
$$\begin{array}{l}\mathrm{CHO}\\ |\\ \mathrm{CO}\\ |\\ -\!\!\!+\!\!-\mathrm{H}\\ |\end{array}$$

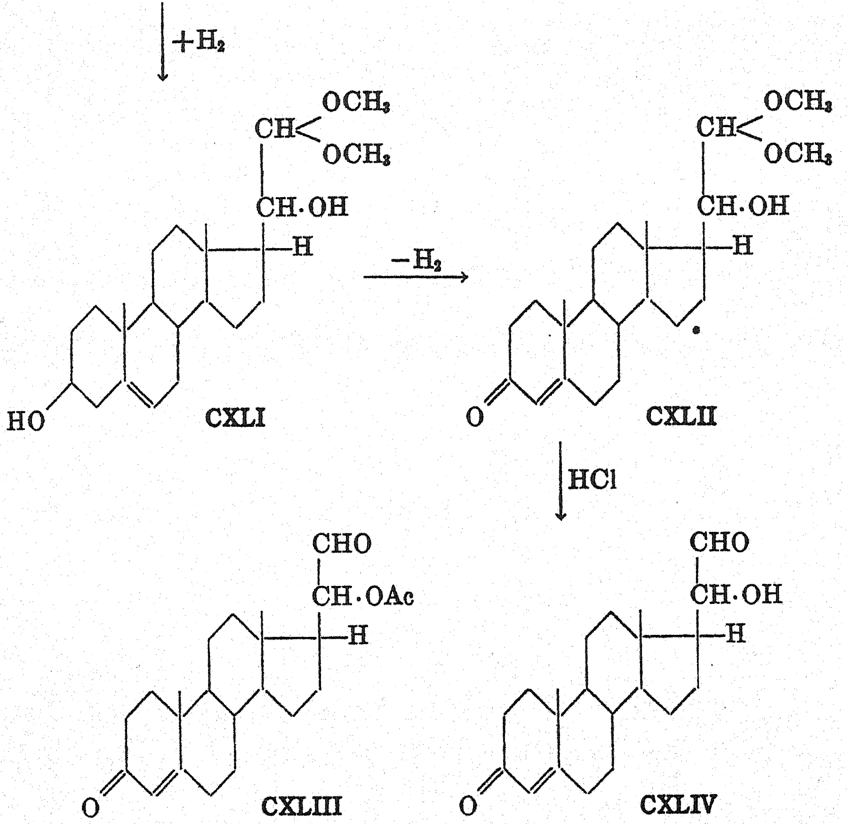


$$\begin{array}{l}\mathrm{CHO}\\ |\\ \mathrm{CH\cdot OH}\\ |\\ -\!\!\!+\!\!-\mathrm{H}\\ |\end{array}$$

from the diazoketones (**CXV, CXVI**), has been carried out as follows:


CHN₂
CO
H
HO
CXV
−H₂
CHN₂
CO
H
O
CXVI
HCl
HCl
CH₂Cl
CO
H
HO
CXXXI
−H₂
CH₂Cl
CO
H
O
CXXXII


pyridine
pyridine
CXXXIII
CXXXIV
p-nitroso-dimethylaniline
p-nitroso-dimethylaniline
CXXXV
CXXXVI
HCl
HCl
CXXXVII
CXXXVIII
CH₃·OH
CH₃·OH
−H₂
CXXXIX
CXL



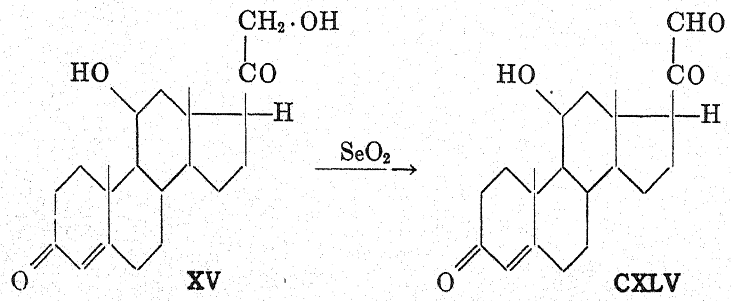
CXLI **CXLII** **CXLIII** **CXLIV**

The diazoketones (**CXV**, **CXVI**) by treatment with hydrochloric acid gave the 21-chloro-compound (**CXXXI**) and 21-chloroprogesterone (**CXXXII**), the latter also being obtained from (**CXXXI**) by Oppenauer oxidation. The chloroketones were converted by warm pyridine into the quaternary pyridinium salts (**CXXXIII**, **CXXXIV**), which with *p*-nitrosodimethylaniline, method of Kröhnke *et al.* (186), furnished the nitrones (**CXXXV**, **CXXXVI**); these were hydrolyzed by shaking with dilute hydrochloric acid at 20° to give the free keto-aldehydes (**CXXXVII**, **CXXXVIII**), both of which crystallized as hydrates (187). The aldehyde (**CXXXVIII**) in the Everse-de Fremery test gave a positive result with 2.5 mg. daily; at 1 mg. daily the result was negative, and the compound in this method of assay is thus less active than corticosterone (187).

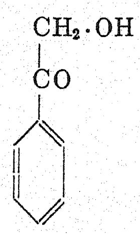
Both the ketoaldehydes (**CXXXVII**, **CXXXVIII**) afforded crystalline dimethylacetals (**CXXXIX**, **CXL**) with methanol in the presence of hydrogen chloride, the former (**CXXXIX**) being converted into the latter (**CXL**) by Oppenauer oxidation. By reduction with aluminum *iso*propylate by the

Meerwein-Ponndorf method (188, 189), (**CXXXIX**) afforded a mixture of two stereoisomeric dihydroxydimethylacetals, the main product (**CXLI**) being isolated in a state of purity (190). By oxidation, most simply using the Oppenauer method, (**CXLI**) was converted into the dimethylacetal (**CXLII**), from which hydrochloric acid liberated the hydroxyaldehyde (**CXLIV**); both (**CXLIV**) and its 20-monoacetate (**CXLIII**) were obtained in the crystalline state (190), which probably represents di- or trimeric forms.

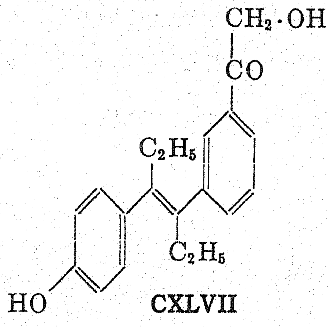
The preparation, in an impure condition, of the ketoaldehyde (**CXLV**) from corticosterone (**XV**) by oxidation with selenium dioxide has been reported by Mason (6); the product was less active than corticosterone in some test method not specified.



XV **CXLV**



CXLVI



CXLVII

The preparation and biological examination of a number of non-steroid α-ketols, $R \cdot CO \cdot CH_2OH$, has been reported by Linnell and Roushdi (191). Of these only benzoylcarbinol (**CXLVI**) and the compound (**CXLVII**), which is reminiscent structurally of stilbestrol, showed any activity; in the life maintenance test in young rats (**CXLVI**) possessed about 1/2500 and (**CXLVII**) about 1/200 of the activity of desoxycorticosterone acetate.

V. Amorphous Fractions with Higher Activity

It is found, particularly if the dog method of assay is used as a criterion, that the sum of the activities of all the crystalline substances isolated from a given quantity of glands represents only a fraction of the original total activity, the greater part of which remains in the mother liquors.

Pfiffner *et al.* (192) and Wintersteiner and Pfiffner (57) found that, after removal of the crystallizable substances from good concentrates, amorphous fractions remained which contained the principal part of the activity, since they were active in the test in dogs at a daily dose of 0.0025 mg. per kg.[17] Under the same conditions 0.1 mg of 17-hydroxy-11-dehydrocorticosterone (VIII) was completely inactive. The amorphous material was thus considerably more than 40 times as active as (VIII). Mason (6) and Kendall (194) described amorphous fractions of which 0.02–0.04 mg. per day sufficed to maintain a 20 kg. adrenalectomized dog in good condition. Under the same conditions, the following quantities of various crystalline substances were required:

Desoxycorticosterone acetate (**CXVII**)	0.1 mg.
Desoxycorticosterone (**XXII**)	0.3 mg.
Corticosterone (**XV**)	2 mg.
17-Hydroxy-11-dehydrocorticosterone (**VIII**)	7–10 mg.

The amorphous fraction was thus 2.5–5 times as active as desoxycorticosterone acetate, and contained *ca.* 90 per cent of the activity of the last concentrate and about 50 per cent of that of the original extract. The activity disappeared on treatment with acids or alkali, or sometimes spontaneously for no apparent reason. The solubility and analytical composition of this purified amorphous fraction corresponded with those of the compounds of the $C_{21}O_5$ group.

Results in rats are much less clear and to some degree contradictory. Reichstein (59, 2) described the preparation of amorphous concentrates which were 3–4 times as active as corticosterone in the Everse-de Fremery test, and were thus about one fourth as active as desoxycorticosterone. Kuizenga and Cartland (53) reported the preparation of concentrates which assayed at 20–30 rat units per mg. in the survival test in young rats. Corticosterone assayed at 6 rat units per mg. and desoxycorticosterone acetate at 35 units per mg.; this amorphous concentrate was thus a little less active than desoxycorticosterone acetate.

On the other hand, Grollman (195, 100) described a simple method for the preparation of adrenal extracts, which (calculated on the basis of the dry residue) showed 10 times the activity of desoxycorticosterone acetate[18] in his modification of the survival and growth test in young rats (99). Grollman also reported the isolation from his concentrates of small quantities of crystals, m.p. 182–183° (100), which were 10 times as active as his amorphous concentrate, and thus 100 times as active as desoxycorticosterone acetate; a microphotograph of the crystals, a crystallographic description, and an approximate analysis was given (195); according to the latter the substance could be an incompletely pure member of the $C_{21}O_5$ group. Grollman did not claim that he was dealing with a pure compound, but mentioned that the activity was unaltered by further fractionation. He nevertheless believed the apparent high activity to be due to a single substance, for which he desired to reserve the description of "the hormone" of the adrenal cortex. The isolation of the crystals appears, unfortunately, not always to be reproducible, and neither identification nor constitutional study has yet been possible.

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[17] There seems to be no direct relation between the weight of a dog and the minimal dose of hormone required (193).

Hartman and Spoor (196) by fractionation of adrenal extracts obtained a concentrate distinguished by its strong action on sodium retention, and termed it the "sodium-factor." For the remaining material, which had increased activity in regard to survival but no effect on sodium retention, they wished to reserve the description "cortin"; Hartman also believed that the main activity of this material was associated with a single substance.

On account of the contradictory results in rats, the writers do not attempt to draw any conclusions from work performed with this animal. Nevertheless, it appears probable that suitably prepared concentrates from adrenal extracts contain an unknown compound, which is considerably more active in the test in dogs that any of the pure crystalline substances yet isolated. As indicated by analysis, the amorphous fraction of Kendall appears to consist principally of substances of the $C_{21}O_5$ group, a conclusion supported by the solubility and the technique of fractionation; desoxycorticosterone, the only substance to approach the activity of the amorphous fraction in the dog test, cannot be present. Certain biological results point in the same direction, but these must be judged with reserve, because two or more substances together may produce a synergistic effect.[19] Again, a higher degree of activity may be occasioned by "activators" such as the "X-substance" of Laqueur *et al.* (197) present in testicular extracts.[20]

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[18] The "maintenance dose" of desoxycorticosterone acetate was found to be 1 mg. per day per rat, a value considerably higher than that found by other workers. Grollman (100) also found oral administration of DOCA in the drinking water to be as effective as parenteral administration, in contrast to the findings of other workers. Thus Kuizenga *et al.* (82) in their test in rats found for desoxycorticosterone acetate 35 rat units per mg. by injection, but less than 1 rat unit per mg. when administered by stomach-tube. It is possible that higher activity is obtained by administration in the drinking water because part of the substance can be resorbed in the mouth, and especially through the tongue, so avoiding passage through the liver.

Whether such amorphous fractions contain one or more very highly active unknown compounds is a question only to be answered after actual isolation of such substances. Until then it is a matter of personal choice as to whether one refers to "*the* hormone" of the adrenal gland, reserving this title for some yet unknown substance, or prefers to speak of several adrenal cortical hormones, reckoning as such all those substances shown to be active in one or other of the methods of assay. The former choice is that of Hartman and of Grollman, the latter that of Kendall and the present writers.

VI. CONSTITUTION AND BIOLOGICAL ACTIVITY

Only a few facts, which appear to the reviewers to be the most important, can be considered here; for a fuller discussion of physiological work see the reviews of Kendall (11) and Pfiffner (14) and the thesis of Monnet (16).

In Table II are collected the relative activities as determined in eight different methods of assay of the six active pure crystalline substances isolated from the adrenal gland; for comparison, the activities of progesterone and of highly active amorphous fractions are included. The sign + denotes activity, and the sign − signifies negative Na^+ and Cl^- retention, *i.e.* increased excretion.

Disregarding progesterone (**XXIV**) (which only displays very weak activity in a few of the methods of assay) and leaving out of consideration (**VII**) (which is insufficiently investigated, and may possibly exhibit some difference in behavior), the table shows that the assay methods fall into two groups. In the first group, *a–d*, the activity displayed decreases with increasing oxygen-content,[21] in the second group, *e–g*, the inverse is the case. Progestational activity is given in the last column, *h*; apart from progesterone (**XXIV**), only desoxycorticosterone (**XXII**) and its acetate (**CXVII**) show progestational activity, which is of a low order.[22] The activity of the "amorphous fractions" in most methods of assay is such as

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[19] Ingle (98) found that adrenalectomized rats treated with a mixture of desoxycorticosterone (**XXII**) and 17-hydroxydehydrocorticosterone (**VIII**) performed more work than animals treated with an equal quantity of either substance alone.

[20] Some support for this suggestion is to be found in the observation of Miescher *et al.* (96) that palmitic acid increased the activity of desoxycorticosterone.

[21] Increasing oxygen-content is paralleled by increasing solubility in water, but this is not to be regarded as the sole cause of the decrease in activity.

[22] According to McGinty (198), this compound is inactive on intra-uterine application.

TABLE II

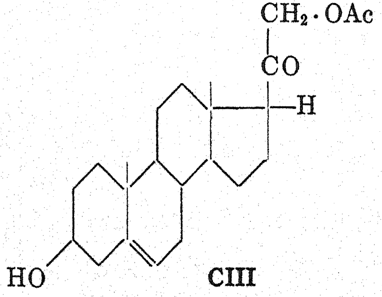
Relative Activities of Crystalline Compounds in Different Methods of Assay

Substance	Group according to oxygen content	Solubility in water on distribution between water and benzene	*a*	*b*	*c*	*d*	*e*	*f*	*g*	*h*
			Survival test in rats[a]	Test in adrenalectomized dogs[d]	Na^+ and Cl^- retention in normal dogs[f]	Everse-de Fremery test[g]	Ingle's test[h]	Diabetogenic action[i]	Anti-insulin test[j]	Clauberg test; progestational activity[k]
Progesterone (**XXIV**)	$C_{21}O_2$	nearly 0	+[b]	?	+	0	0	0	0	++++
Desoxycorticosterone acetate (**CXVII**)	$C_{21}O_3$	nearly 0	+++++	++++	++++	+++++	+ or 0	0	0	+
Desoxycorticosterone (**XXII**)	$C_{21}O_3$	+	++++	+++	++++	++++	+ or 0	0	0	+
17-Hydroxy-11-desoxycorticosterone (**XI**)	$C_{21}O_4$	++	++ (?)[l]	?	?	++[l]	+ or 0[l]	?	0[l]	
Corticosterone (**XV**)	$C_{21}O_4$	++	++	++	++	++	+++	++	++	0
11-Dehydrocorticosterone (**XVI**)	$C_{21}O_4$	++	++	++		?	+++	++		0
17-Hydroxy-corticosterone (**VII**)	$C_{21}O_5$	+++	+	+	− − − −	+	++++		+++	0
17-Hydroxy-11-dehydrocorticosterone (**VIII**)	$C_{21}O_5$	+++	+	+	− − − −	+	++++	++	+++	0
"Amorphous fraction"	$C_{21}O_5$(?)	+++	+++++[c]	+++++[e]	+(?)[e]	+++[m]	+(?)	0(?)[e]	?	0

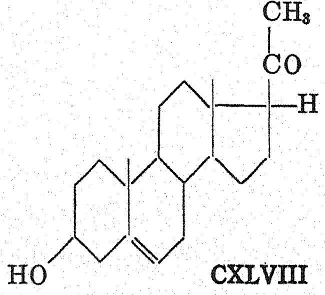
[a] Based on results of Kuizenga *et al.* (82).
[b] Compare 108, 109, 110.
[c] Amorphous fraction of Kuizenga and Cartland (53).
[d] Based on results of Kendall *et al.* (3, 6, 54, 194).
[e] Amorphous fraction of Kendall *et al.* (6, 194).
[f] Based on results of Thorn *et al.* (65, 112).
[g] Based on results of Waterman *et al.* (97) and figures reported by Reichstein (2, and this review).
[h] Based on results of Ingle (60, 98, 61, 111).
[i] Based on results of Long *et al.* (26).
[j] Based on results of Grattan and Jensen (28).
[k] Compare 96, 102, 103.
[l] Activity of the acetate.
[m] Amorphous fraction of Reichstein (59, 2).

completely to break the vertical sequence; but so long as it remains uncertain whether their activity is caused by a single substance or by several substances, no conclusion can be drawn from this singular fact.[23]

For activity in regard to carbohydrate metabolism (test method *e* may probably be included here with methods *f* and *g*), the presence of an oxygen atom at C_{11} appears essential. A hydroxyl group at C_{17} increases the effect, but only so long as the oxygen atom at C_{11} is present. Whether the presence of oxygen atoms at other positions also produces a favorable effect has been little investigated.[24] The influence on carbohydrate metabolism is not completely specific, since the physiologically potent cardiac glycosides strophanthidin, ouabain, and digitalin, of which the former and the latter certainly contain no oxygen atom at C_{11}, are able in small doses not only to lengthen the life of adrenalectomized rats but also to exhibit some anti-insulin activity (199, 200, 201). It may be mentioned that stilbestrol possesses diabetogenic activity (202).



CIII



CXLVIII

It has been stated for some time that desoxycorticosterone (**XXII**) is the simplest substance which is able to reproduce in numerous methods of assay the effects of adrenal extracts. It was also thought that any alteration of the molecule caused complete inactivation, but observations have been made which appear to call for modification of this view. Thus Kuizenga and Cartland (53) reported that *allo*pregnane-3(α):11:17(β):21-tetrol-20-one (**III**) was active in the survival test in rats; and Selye (172) has stated that Δ^5-pregnene-3(β):21-diol-20-one as the 21-monoacetate (**CIII**) was almost as active as desoxycorticosterone acetate in the survival test in rats, while Δ^5-pregnene-3(β)-ol-20-one (**CXLVIII**) was also active.

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[23] The amorphous fractions of different workers are naturally not comparable among themselves.

[24] Δ^4-Pregnene-6:21-diol-3:20-dione as the diacetate is inactive in Ingle's test (184).

In this connection, the activity of strophanthidin (mentioned above[25]) and of the synthetic non-steroid compound (**CXLVII**) should not be overlooked.

As to the mechanism whereby the adrenal cortical hormones exert their action, practically nothing is known. Verzár has recently been able to adduce support for his theory (9) that these hormones are involved in phosphorylation processes; he has shown (207) that the activity *in vitro* of crushed muscle tissue from adrenalectomized rats, in which the phosphorylative degradation of glycogen is strongly retarded, can be restored to normal by small quantities of desoxycorticosterone acetate.

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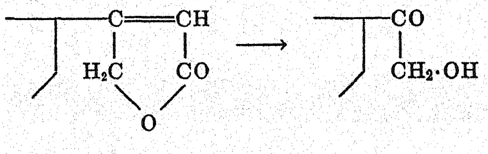
[25] The conversion of the unsaturated lactone structure (203, 204) of the cardiac glycosides into the α-ketol group characteristic of the adrenal cortical hormones by β-oxidation is an obvious theoretical possibility; the reverse reaction has been realised *in vitro* (205, 206).



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